PCT

WORLD INTELLECT
Int

WO    9604000A1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: PEPTIDE-BASED NUCLEIC ACID MIMICS (PENAMs)

(57) Abstract

The present invention provides novel nucleic acid mimics (termed "PENAMs") comprising a peptidic backbone and nucleotidic sidechains; the sidechains being oriented in such a way that the PENAM is homomorphous to target nucleic acids with which it can effectively hydrogen bond. Homomorphism is achieved by the incorporation of unusual stereochemical centers, including D-chiral centers and quasi-chiral centers, into the peptidic backbone. The PENAMs are useful for targeting nucleic acid sequences in order to modulate their activity in an "antisense" manner. Targeting can also be used to detect, isolate or modify target nucleic acids.

1

## PEPTIDE-BASED NUCLEIC ACID MIMICS (PENAMs)

### Field of the Invention

The invention generally relates to compounds that
10  are capable of binding to nucleic acids in a
sequence-specific manner. More specifically, the
invention provides novel chimeric molecules having a
backbone that resembles a polypeptide but side chains
that resemble the base portions of nucleic acids. The
15  invention also relates to the construction of such
chimeric molecules; and to their use in targeting
complementary nucleic acids.

### Background

20  Nucleic acids can be targeted by virtue of the
ability of "antisense" nucleotidic bases to engage in
hydrogen bonding with complementary bases of a target
nucleic acid, thereby modulating expression of the
target. The use of sequence-specific recognition of
25  pathogenic genes to regulate or inhibit their expression
in an antisense manner, using short synthetic
complementary oligo(deoxy)nucleotides ("ODNs") and their
analogs has been an attractive strategy for rational drug
design [1, 2]. [Throughout this document, arabic numbers
30  in square brackets refer to one or more references cited
in the the list of References below.]

A simple illustration of such a technique would be
the administration of a DNA oligomer complementary to a
target mRNA that encodes a protein necessary for the
35  progression of infection of an agent such as a virus. In
such a case, the administered DNA might bind to the
target mRNA transcript, resulting in translation arrest,

2

thereby blocking the synthesis of a protein critical to infection. Hybridization to a nascent mRNA may lead to premature termination of transcription. It is also possible to target double-stranded DNA using an appropriate oligomer capable of engaging in the formation of a triple helix via insertion of the oligomer into the major groove of the double-helical DNA.

General approaches to the construction and modification of oligomers useful in antisense therapy have been described in a number of reviews, see, e.g., Miller & Ts'o [1], Matteucci & Bischofberger [2], Cohen & Ghosh [3, 4], Helene & Toulme [5], Stein [6, 7], Uhlmann & Peyman [8], and the papers reviewed therein. In addition, a publicly available bibliography of citations relating to antisense oligonucleotides is maintained by Dr. Leo Lee at the Frederick Cancer Research Facility in Frederick, Maryland, which references are hereby incorporated herein.

Although there have been some promising results from the use of antisense ODNs in cell culture, suggesting the potential of this methodology for inhibiting gene expression, ODNs are subject to two significant limitations: (i) relatively poor transport across cellular membranes and (ii) sensitivity to cellular nucleases [4].

There have been a number of attempts to circumvent these problems by modifying the oligomers. For example, since nucleases are known to attack the phosphodiester linkage, a number of modified oligonucleotides have been prepared which contain alternate linkages, such as methylphosphonates (wherein a phosphorous-linked oxygen has been replaced with a methyl group), phosphorothioates (wherein a phosphorous-linked oxygen has been replaced with a sulfur group), and various amidates (wherein $NH_2$ or an organic amine replaces one of these oxygen atoms); see e.g., the work primarily reviewed by Uhlmann & Peyman [8] and others [9-14].

3

Other efforts have been focused on changing or replacing phosphodiester groups or other parts of the sugar phosphate backbone by variety of linkages which include, for example: carbamate derivatives and

5   morpholine derived carbamate derivatives [15, 16]; polyvinylic derivatives [17, 18]; polyphosphate derivatives [19]; polylysine derivatives and polyethylene amine derivatives [20]; peptidic derivatives [21-33]; nylon-based derivatives [34, 35]; and polyamide

10  derivatives [36-42].

In some of these cases the modified oligomers were capable of hybridizing to the target nucleic acid, but exhibited shortcomings related to stability, sensitivity to degradative enzymes and/or cellular uptake. In some

15  other cases, the modified oligomers were relatively stable, and fairly resistant to degradative enzymes (due to the unusual linkages), but hybridization between these modified oligomers and their nucleic acid targets was sub-optimal. (As described below, we believe that some

20  of these shortcomings may be due to the electrostatic and/or spatial properties of these oligomers.)

Accordingly, there remains a need for molecules that are: (i) relatively easily taken up and transported by cells; (ii) relatively stable and resistant to cellular

25  degradative enzymes; and (iii) effective at binding to complementary nucleic acids.


Summary of the Invention

It is a primary object of the present invention to

30  provide a molecule that is able to function like a nucleic acid in antisense targeting but which overcomes certain of the disadvantages associated with the use of oligonucleotides (ODNs) for antisense targeting.

The present invention provides a chimeric molecule

35  referred to as a "peptide-based nucleic acid mimic" (or "PENAM"). These chimeric molecules have a peptide-like backbone with side chains that contain bases resembling

4

those normally found in nucleic acids.  The bases on the
nucleic side chains of the PENAM allow the molecule to
engage in hydrogen bonding with a target nucleic acid
having a substantially complementary sequence of bases;
5    or to engage in triple helix formation, as described
below.

The chimeric PENAMs of the present invention also
have    an    unusual    stereochemical    composition    that
facilitates binding to the target nucleic acid.    In
10   particular, the PENAMs of the present invention have a
peptidic    backbone    that    incorporates    unusual    chiral
centers    (including    D-chiral    centers    and    quasi-chiral
centers) that can be used to orient the nucleic side
chains in such a way that the nucleotidic bases are
15   spatially homomorphic to bases in targeted nucleic acids.
The ability to enhance binding by spatial homomorphism is
especially    significant    given    that    hydrogen    bonding
interactions between biomolecules typically depend on an
aggregation of many relatively weak bonds.    The PENAMs
20   are also much less susceptible to electrostatic charge
repulsion (because of the replacement of the normally
charged backbone).    Also, by virtue of their unusual
structural and stereochemical features, the PENAMs of the
present invention are resistant to degradative enzymes
25   that    are    expected    to    be    present    in    most    biological
systems.    In particular, the PENAMs do not possess the
phosphodiester backbone which is the standard target of
the nucleases.  Moreover, the peptidic backbone is unlike
that    of    naturally    occurring    peptides    because    of    the
30   presence of unusual chiral centers including D-chiral
centers and/or quasi-chiral centers.

In addition, the peptidic backbone is assembled from
monomers (termed "NuAA monomers", described below) which
can    themselves    be    conveniently    linked    using    peptide
35   synthetic techniques.  These building block NuAA monomers
thus    allow    for    the    rapid    production    of    a    variety    of
"antisense" oligomers (exhibiting differing sequences for

5

targeting particular complementary nucleic acids), via an automated peptide synthesizer.

Thus, one aspect of the present invention is a novel chimeric oligomer having bases that resemble those in ODNs, but in which the entire ribose- or deoxyribose-phosphate backbone has been replaced with a peptidic backbone that has been designed to incorporate unusual chiral centers including D-chiral centers and/or quasi-chiral centers. The resulting peptidic backbone is capable of orienting the side chains such that the nucleic acid mimic can effectively bind to a complementary target nucleic acid. These novel chimeras, termed "peptide-based nucleic acid mimics" (or "PENAMs") are stable under physiological conditions and resistant to degradative enzymes, and are electrostatically and spatially designed for enhanced transport across cellular membranes and effective binding to target nucleic acids.

One aspect of the invention is a composition useful in targeting a nucleic acid comprising a stereochemically-selected population of peptide-based nucleic acid mimics (PENAMs) each member of which comprises a sequence of at least about 4 NuAA monomers, wherein at least one of said PENAMs is a homomorphically-preferred PENAM comprising a D-chiral center or a quasi-chiral and wherein said homomorphically-preferred PENAM makes up at least about 10% of the stereochemically-selected population of PENAMs. The NuAA monomers are each of the following formula:
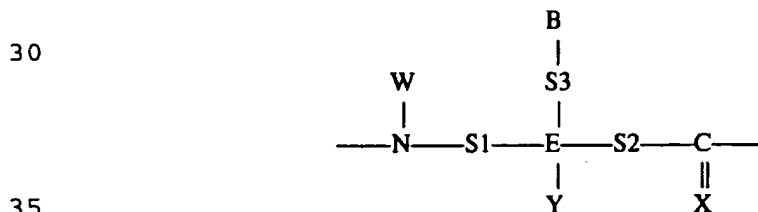
$$
\begin{array}{ccc}
 & B & \\
 & | & \\
W & S3 & \\
| & | & \\
\text{—N—S1—E—S2—C—} \\
| & \| \\
Y & X
\end{array}
$$

wherein:

E is carbon (C) or nitrogen (N);

W is hydrogen or a spacer group;

6

Y is hydrogen or a spacer group (when E is carbon),
or Y is a lone pair of electrons (when E is nitrogen);

S1 is a bond or a first spacer group;

S2 is a bond or a second spacer group;

S3 is a bond or a third spacer group;

X is oxygen (O) or sulfur (S);

B is a base segment comprising a nucleotidic base or
an analog thereof; and

N is nitrogen.

Preferably, the homomorphically-preferred PENAM
makes up at least about 30% of the stereochemically-
selected population of PENAMs; more preferably at least
about 50%; still more preferably at least about 80%.

Another aspect of the invention is free NuAA monomer
that is a precursor of a NuAA monomeric subunit
comprising a quasi-chiral center wherein said NuAA
monomeric subunit comprising a quasi-chiral center is of
the following formula:

$$
\begin{array}{ccccc}
 &  & B &  &  \\
 &  & | &  &  \\
 & W & S3 &  &  \\
 & | & | &  &  \\
\text{—N} & \text{—S1—} & \text{E} & \text{—S2—} & \text{C—} \\
 &  & | &  & \| \\
 &  & Y &  & X \\
\end{array}
$$

wherein:

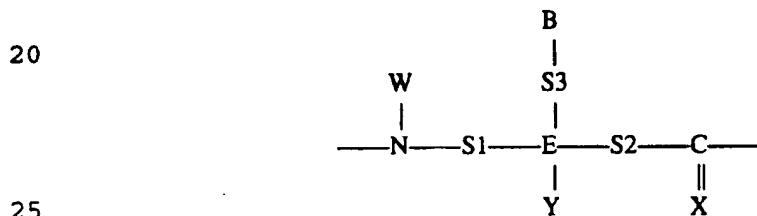E is nitrogen (N);

W is hydrogen or a spacer group;

Y is hydrogen or a spacer group (when E is carbon),
or Y is a lone pair of electrons (when E is nitrogen);

S1 is a bond or a first spacer group;

S2 is a bond or a second spacer group;

S3 is a bond or a third spacer group;

X is oxygen (O) or sulfur (S);

B is a base segment comprising a nucleotidic base or
an analog thereof; and

N is nitrogen;

wherein said free NuAA monomer is of the same formula except that it has an amine group or a protected amine group at its N-terminus and it has a carboxyl group or a protected carboxyl group at its C-terminus.

5     Another aspect of the invention is a free NuAA monomer that is a precursor of a NuAA monomeric subunit comprising a D-chiral center wherein said NuAA monomeric subunit comprising a D-chiral center is of the following formula:

10

$$
\begin{array}{ccccc}
 & & B & & \\
 & & | & & \\
 & W & S3 & & \\
 & | & | & & \\
\text{---}N\text{---}S1\text{---}E\text{---}S2\text{---}C\text{---} \\
 & & | & & \| \\
 & & Y & & X
\end{array}
$$

15
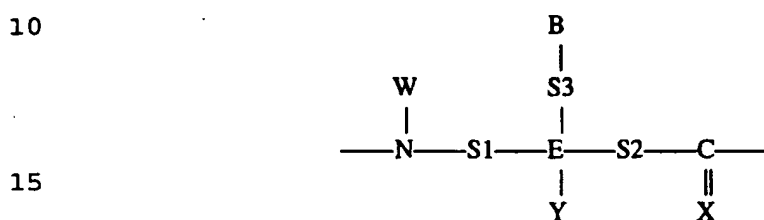
wherein:

E is carbon (C);

20     W is hydrogen or a spacer group;

Y is hydrogen or a spacer group;

S1 is a bond or a first spacer group;

S2 is a bond or a second spacer group;

S3 is a third spacer group with a backbone of at

25 least two atoms;

X is oxygen (O) or sulfur (S);

B is a base segment comprising a nucleotidic base or an analog thereof; and

N is nitrogen;

30     and wherein at least one of S1 and S2 is a spacer group, and wherein S3 is a spacer group with a backbone of at least two atoms;

wherein said free NuAA monomer is of the same formula except that it has an amine group or a protected

35 amine group at its N-terminus and it has a carboxyl group or a protected carboxyl group at its C-terminus.

Another aspect of the invention is a method of preparing a PENAM composition for targeting a target nucleic acid comprising: (a) providing at least about 4

8

NuAA monomers, preferably at least about 6 NuAA monomers, wherein at least one of said NuAA monomers comprises a D-chiral center or a quasi-chiral center; and ( b ) synthesizing an oligomer of the monomers such that the

5   arrangement of bases in the oligomer is substantially complementary to a sequence of nucleotidic bases in a portion of the target nucleic acid. Preferably step (a) involves providing at least about 6 NuAA monomers, more preferably at least about 10 NuAA monomers, still more

10  preferably at least about 14 NuAA monomers. The NuAA monomers can be linked to each other directly forming peptide bonds, or indirectly via an intervening residue such as an amino acid residue.

Another aspect of the invention is a method of

15  targeting a target nucleic acid comprising: (a) providing a PENAM composition; and (b) contacting said PENAM composition with the target nucleic acid.

Another aspect of the invention is a method of modulating a target nucleic acid in an antisense manner

20  comprising: (a) providing a PENAM composition wherein the sequence of bases in said NuAA monomers is substantially complementary to a sequence of bases in the target nucleic acid; and (b) contacting said PENAM composition with the target nucleic acid.

25  Another aspect of the invention is a method of modifying a target nucleic acid comprising: (a) providing a PENAM composition wherein said homomorphically-preferred PENAM further comprises a target modifying group; and (b) contacting said PENAM composition with the

30  target nucleic acid.

Another aspect of the invention is a method of detecting a target nucleic acid comprising: (a) providing a PENAM composition wherein the sequence of bases in said NuAA monomers is substantially complementary to a

35  sequence of bases in the target nucleic acid; and (b) contacting said PENAM composition with the target nucleic acid; and (c) detecting a target complex comprising said

9

nucleic acid mimic and said target nucleic acid or detecting a modification in the target nucleic acid.

Another aspect of the invention is a method of isolating a target nucleic acid comprising: (a) providing
5   a PENAM composition wherein the sequence of bases in said NuAA monomers is substantially complementary to a sequence of bases in the target nucleic acid; and (b) contacting said PENAM composition with the target nucleic acid; and (c) isolating PENAMs bound to said target
10  nucleic acid or isolating a modified target nucleic acid.


## Brief Description of the Drawings

Figure 1 is a comparison of monomers in oligo(deoxy)nucleotides    (such    as    DNA    and    RNA,
15  collectively referred to as "ODNs"), and the NuAA monomers that form the peptide nucleic acid mimics (PENAMs) of the present invention.

Figure 2 is an illustrative example of molecular modelling of an (L)-PENAM (Version I, constructed from L-
20  NuAA monomers) and a (D/L)-PENAM (Version II, constructed from alternating D- and L-NuAA monomers), shown in beta-sheet conformation in both a "side view" (Figure 2a) and a "top view" (Figure 2b).

Figure 3 is an illustrative example of molecular
25  modelling comparisons of various PENAMs (differing in the length of their "S2" spacer group), each bound to a complementary strand of target DNA. The S2 spacer group positions are occupied by bonds (Figure 3a), methylene groups (Figure 3b), ethylene groups (Figure 3c), or
30  propylene groups (Figure 3d); as described in Example 2 below. Figure 3e is a corresponding model illustrating the interaction between two complementary DNA strands.

Figures 4a and 4b are illustrative examples of the retrosynthesis of PENAMs and NuAA monomers from
35  $\alpha$-amino-$\alpha$-carboxylic acids.

Figure 5 is an illustrative example of the synthesis´ of a NuAA monomer and a PENAM starting from (D/L)-methionine.

Figure 6 illustrates a synthetic strategy for
5  preparing NuAA monomers containing various nucleic bases (A, T, G, C, U).

Figure 7 is an illustrative example of the retrosynthesis of PENAMs and NuAA monomers from $\alpha$-amino-$\omega$, $\alpha$-dicarboxylic acids.

10  Figure 8 is an illustrative example of the retrosynthesis of aza-NuAA monomers from aza-amino acids.

Detailed Description of the Invention

15  Definitions

The following definitions are intended to supplement, not to replace, the detailed descriptions of these and other aspects of the invention which are described herein.

20  A "peptide-based nucleic acid mimic" (or "PENAM", sometimes referred to as a "peptide nucleic acid mimic" or "nucleic acid mimic"), refers to a molecule having a peptidic backbone with side chains having nucleotidic bases that are capable of engaging in hydrogen bonding
25  with a nucleic acid having a complementary sequence of bases. The PENAMs of the present invention are assembled from NuAA monomers, but may also contain other chemical groups such as "target modifying groups", natural amino acids or peptides, and other groups as described below.
30  Such additional groups may be located within an individual NuAA monomer, or may occupy positions either between NuAA monomers or outside of a string of NuAA monomers. The term "monomer" is used to refer to individual molecules that can be incorporated by
35  polymerization into larger molecules; and is also used to refer to the corresponding monomeric subunits (i.e. after incorporation into the larger molecule).

11

The term "complementary" indicates that a particular sequence of bases is able to pair (as in Watson-Crick base-pairing) with corresponding bases in a given target sequence.   The term "substantially complementary"

5  indicates that at least about 80% of the bases in a particular sequence are able to engage in base-pairing with corresponding bases of the target sequence.  The term "partially complementary" indicates that at least about 60% of the bases in a particular sequence are able

10  to engage in base-pairing with corresponding bases of the target sequence.

A "target nucleic acid" refers to a nucleic acid comprising a sequence of bases that is at least partially complementary to a sequence of base groups on a targeting

15  PENAM.

The "peptidic backbone" refers to the portion of a PENAM that comprises peptide bonds derived from the aminoacyl portions of the NuAA monomers and/or intervening aminoacyl monomers such as natural or

20  unnatural amino acids.

The "nucleic side chains" refer to portions of the PENAM that comprise nucleotidic bases which are capable of engaging in hydrogen bonding with corresponding bases on a target nucleic acid.

25      A "nucleic aminoacyl monomer" (or "nucleic aminoacyl monomeric subunit") refers to a monomeric subunit of the following formula:
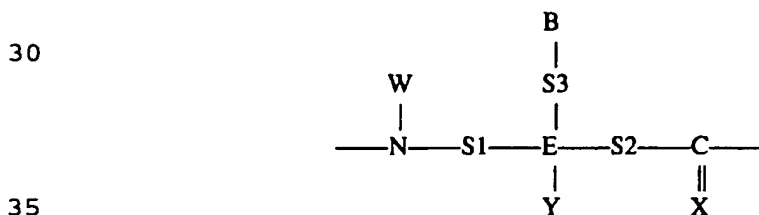
$$
\begin{array}{ccc}
 & B & \\
 & | & \\
W & S3 & \\
| & | & \\
\text{---N---S1---E---S2---C---} & & \\
| & \| & \\
Y & X &
\end{array}
$$

wherein:

E is carbon (C) or nitrogen (N);

W is hydrogen or a spacer group;

12

Y is hydrogen or a spacer group (when E is carbon),
or a lone pair of electrons (when E is nitrogen);

S1 is a bond or a first spacer group;

S2 is a bond or a second spacer group;

5    S3 is a bond or a third spacer group;

X is oxygen (O) or sulfur (S);

B is a base segment comprising a nucleotidic base or
an analog thereof; and

N is nitrogen.

10   A "free nucleic amino acid monomer" (or "free NuAA
monomer") refers to a precursor of a nucleic aminoacyl
monomer.  The precursor monomer is essentially of the
same formula as shown above except that the N-terminus
(i.e. the N with a free bond in the formula) is in the
15   form of an amine or protected amine (e.g. a carbamate);
and the C-terminus (i.e. the C with a free bond in the
formula) is in the form of a carboxylic acid or, e.g., an
ester.

"NuAA monomer" is used to refer to both free NuAA
20   monomers and to corresponding NuAA monomeric subunits
after incorporation into a larger molecule.

An "aza-NuAA monomer" refers to a NuAA monomer of
the formula shown above in which E is nitrogen.  The
nitrogen at position E forms a "quasi-chiral center" as
25   discussed herein.

An "L-NuAA monomer" refers to a NuAA monomer in
which E is carbon and is a chiral center that is in a
stereochemical configuration corresponding to that of an
analogous L-amino acid (i.e., an L-amino acid having an
30   R group in place of S3-B and having carbonyl and amide
groups oriented in the directions as shown in the formula
above).

A "D-NuAA monomer" refers to a NuAA monomer in which
E is carbon and is a chiral center that is in a
35   stereochemical configuration corresponding to that of an
analogous D-amino acid (i.e., a D-amino acid having an R
group in place of S3-B and having carbonyl and amide

13

groups oriented in the directions as shown in the formula above). The carbon at position E in a D-NuAA monomer forms a "D-chiral center" as discussed herein.

An "aminoacyl monomer" (or "AA monomer") refers to
5  a structure that resembles a NuAA monomer as shown in the formula above, but lacks the base segment at position B. Thus, AA monomers are derived from amino acids such as a natural or unnatural amino acids. Such AA monomers can be incorporated into PENAMs and can (either singly or in
10 strings) occupy positions between NuAA monomers. Such AA monomers can, like NuAA monomers, comprise D-, L- and/or quasi-chiral centers.

An "aza-aminoacyl monomer" (or "aza-AA monomer") refers to an analog of an AA monomer in which the chiral
15 center (i.e. the carbon at position E) is replaced with a quasi-chiral center (i.e. a nitrogen at position E).

A "stereochemically-selected population of PENAMs" refers to a population of PENAMs each member of which comprises a sequence of at least about 4 NuAA monomers,
20 wherein at least one of said PENAMs, denoted a "stereochemically-prevalent PENAM" makes up at least about 10% of said population of PENAMs, preferably at least about 30%, more preferably at least about 50%, still more preferably at least about 80%. Generally, the
25 remaining PENAMs in the stereochemically-selected population will be diastereomers of the stereochemically-prevalent PENAM(s); although the population may also include PENAMs of other lengths and/or structure. Stereochemically-selected populations of PENAMs can be
30 most conveniently prepared by utilizing enantiomerically pure starting reagents (preferably at least about 90% enantiomeric excess, more preferably at least 95%, still more preferably at least about 99%), such as D- or L-amino acids, as described herein. Alternatively, one
35 could resolve selected stereochemical intermediates or products, or employ stereo-selective synthetic techniques.

14

A "homomorphically-preferred" PENAM refers to a
PENAM that exhibits structural and stereochemical
properties that facilitate binding between a sequence of
nucleotidic bases in the PENAM and a substantially
5    complementary sequence of bases on a target nucleic acid.
Homomorphically-preferred PENAMs of the present invention
comprise at least one D-chiral or quasi-chiral center.
Homomorphically-preferred PENAMs can comprise various
combinations of monomers including, e.g., NuAA monomers
10   and AA monomers (both of which can contain D- and/or L-
chiral centers); and aza-NuAA monomers and aza-AA
monomers (both of which contain quasi-chiral centers).

The term "flanked" or "flanking" refers to the two
positions in the peptidic backbone immediately adjacent
15   to a particular NuAA monomer.

A "spacer group" refers to a relatively small
chemical group, or series of small chemical groups, that
may be included in the peptidic backbone or nucleic side
chains either between adjacent atoms (i.e., at positions
20   S1, S2, and/or S3), or dependent from atoms (i.e. at W
and/or Y) in the formula shown above; and that is
unlikely to inhibit hydrogen bonding between the
targeting PENAM and the target nucleic acid.    These
spacer groups contain a backbone of 1-6 atoms (not
25   counting hydrogen atoms), preferably selected from
carbon, nitrogen, oxygen, and sulfur.   Typically, such
spacer groups are substituted or unsubstituted alkyl,
alkenyl, alkynyl groups.   However spacer groups can also
comprise for example: carbonyl (C=O), thiocarbonyl (C=S),
30   amine (NH), substituted amine (NR), amide {C(=O)NH},
substituted amide {C(=O)NR}, carbamate {NHC(=O)O}, urea
{NHC(=O)NH}, thioamide {C(=S)NH}, substituted thioamide
{C(=S)NR}, hydrazine (NH-NH), substituted hydrazine
{N(R)-N(R)}, ether (C-O-C), thioether (C-S-C), disulfide
35   (S-S), sulphone {S(=O)} and sulphoxide (SO2) groups.  The
spacer group backbone can also be substituted with one or
more small chemical groups, for example, small chain

15

alk(ane, ene, yne)s, hydroxyl (OH), alkoxyl (OR), ketone
(COR), aldehyde (CHO), thiol (SH), amine (NH2), and
halogen (F, Br, Cl) groups. A single NuAA monomer may
contain more than one spacer group (at W, Y, S1, S2 and
5   S3 for example) and these need not be identical to each
other.  Currently preferred spacer groups are selected
from methylene, ethylene and propylene groups.

A "base segment" refers to a chemical group
comprising the base portion of a nucleotidic base or an
10  analog thereof, as described below.

A "nucleotidic base" or "nucleic base" refers to a
nitrogenous heterocyclic group typically found in nucleic
acids (such as the purine bases adenine and guanine, or
the pyrimidine bases cytosine, thymine and uracil) or an
15  analog thereof.  An "analog" of a nucleotidic base
refers, for example, to an analog of a purine base in
which the ring substituents are other than those found in
adenine or guanine, or an analog of a pyrimidine base in
which the ring substituents are other than those found in
20  uracil, thymine and cytosine.  Examples of such analogs
are described below and in the art.

A "target modifying group" refers to a chemical
group on a PENAM that is capable, after the PENAM has
hydrogen bonded to a target nucleic acid, of modifying
25  the target nucleic acid.  Such modifying groups include
for example groups which label, reconform, cleave,
covalently bind or intercalate into nucleic acids.
Examples of such target modifying groups are described
below.

30  A nucleic acid "labeling group" refers to a group on
a PENAM which, as a result of the interaction between the
mimic and its target, facilitates labeling and thus
detection of the target nucleic acid.  Examples of such
labeling groups are described below.

35  A nucleic acid "intercalating group" refers to a
group on a PENAM which, as a result of the interaction
between the mimic and its target, intercalates into

16

double helical target nucleic acid.  Examples of such intercalating groups are described below.

A nucleic acid "cleaving group" refers to a group on a PENAM which, as a result of the interaction between the
5 mimic and its target, facilitates the cleavage of the target nucleic acid.  Examples of such cleaving groups are described below.

The phrase "modulating in an antisense manner" refers to modulating the expression and/or activity of a
10 nucleic acid via hydrogen bonding interactions analogous to those described in systems involving antisense oligonucleotides.

The term "homomorphic" (or "homomorphous") refers to the stereochemical fit between a sequence of bases in the
15 targeting portion of a PENAM and a target nucleic acid wherein at least about 1/2 of the bases of the targeting portion of the mimic are positioned to allow hydrogen bonding with complementary bases on the target nucleic acid.  Preferably at least about 2/3 of the bases are so
20 positioned; more preferably at least about 3/4 of the bases are so positioned; most preferably all of the bases are so positioned.

All publications cited herein, including scientific
25 papers, published patent applications and issued patents, are incorporated herein by reference in their entirety.

1.    Nucleic Aminoacyl Monomers (NuAA Monomers)

The peptide-based nucleic acid mimics ("PENAMs") of
30 the present invention comprise a sequence of nucleic aminoacyl monomers ("NuAA monomers").  NuAA monomers are chimeric molecules and/or monomeric subunits of the following formula:

```
                        B
                        |
              W         S3
5             |         |
         ——N——S1——E——S2——C——
                        |        ‖
                        Y        X
```
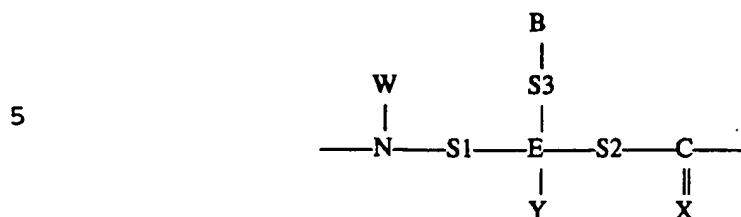
10  wherein:

E is carbon (C) or nitrogen (N);

W is hydrogen or a spacer group;

Y is hydrogen or a spacer group (when E is carbon], or a lone pair of electrons (when E is nitrogen];

15  S1 is a bond or a first spacer group;

S2 is a bond or a second spacer group;

S3 is a bond or a third spacer group;

X is oxygen (O) or sulfur (S);

B is a base segment comprising a nucleotidic base or
20  an analog thereof; and

N is nitrogen.

In free NuAA monomers (i.e. before incorporation into a polymer), the N-terminus (i.e. the N with a free bond in the formula) is in the form of an amine or
25  protected amine (e.g. a carbamate); and the C-terminus (i.e. the C with a free bond in the formula) is in the form of a carboxylic acid or, e.g., an ester.

Nucleotidic bases include the usual bases found in nucleic acids (i.e., the purine bases of adenine and
30  guanine, and the pyrimidine bases of cytosine, thymine and uracil) and analogs thereof. A number of analogs of nucleotidic bases are well known in the art; many of⁻ which have been tested as chemotherapeutic agents. Some of these are described herein; see also, e.g.,
35  Beilstein's Handbuch der Organischen Chemie (Springer Verlag, Berlin), and Chemical Abstracts, which provide references to publications describing the properties and preparation of such compounds, which publications are incorporated herein by reference.

Thus, a large variety of analogs have been described that exhibit properties that may be advantageous in particular targeting schemes. For example, in some cases, it may be desirable to incorporate a base that

5   binds non-specifically at a particular position. The base present in inosine is an example of such a non-specific analog. This can be used to incorporate degeneracy into the PENAM at particular positions which might be particularly useful, for example, in targeting

10  a closely related family of target nucleic acids that are homologous except for one or a few positions in the base sequence. Inosine can pair with all four natural bases although the strength of binding varies: dC > dA > dG/T. See, e.g., [43].

15      Other types of modified bases that may be of particular interest are those which enhance binding affinity. For example, diaminopurine can form three hydrogen bonds with thymine, whereas adenine and thymine form only two [44]. Similarly, pyridopyrimidine bases

20  can be used in place of cytosine to provide stronger base pairing with guanine [45].

Bases can also incorporate any of a variety of "target modifying groups". By way of illustration, base analogs can function as cross-linking moieties. For

25  example, 6-bromo-5,5,-dimethoxyhexanohydrazide can be introduced into the $C^4$ position of cytidine to alkylate and thereby cross link guanosine [46]. $N^4,N^4$-Ethano-5-methyl-cytosine can be used to similar effect [47, 48].

30      Another example of base analogs that can be incorporated into a PENAM are fluorescing analogs such as the base of pyridopyrimidinedeoxynucleosides [45]. Such bases can thus be used as labeling groups.

A wide range of purine and pyrimidine analogs

35  exhibiting various properties is known in the art [see, e.g., references 49-62]. An exemplary but not exhaustive list of such analogs includes: 1-methyladenine,

19

1-methylguanine, 1-methylinosine, 1-methylpseudouracil,
2-methylthio-$N^6$-isopentenyladenine, 2-thiocytosine,
2-methyladenine, 2-methylguanine, 2-thiouracil,
2,2-dimethylguanine, 2,6-diaminopurine-3-methylcytosine,
5  3-(3-amino-3-N-2-carboxypropyl)-uracil-4-acetylcytosine,
4-thiouracil, 5-fluorouracil, 5-iodouracil,
5-bromouracil, 5-methyluracil, 5-methyl-2-thiouracil,
5-methoxyaminomethyl-2-thiouracil, 5-chlorouracil,
5-carboxymethylaminomethyl-2-thiouracil,
10  5-methylaminomethyluracil, 5-carboxyhydroxylmethyluracil,
5-carboxymethylaminomethyluracil, 5-methoxyuracil,
5-methylcytosine, 7-methylguanine, 7-deazaguanine,
7-deazaadenine, β-D-mannosylqueosine,
β-D-galactosylqueosine, dihydrouracil, hypoxanthine,
15  inosine, N-uracil-5-oxyacetic acid methylester,
$N^6$-methyladenine, $N^6$-isopentenyladenine, pseudouracil,
queosine, uracil-5-oxyacetic acid methylester,
uracil-5-oxyacetic acid, and xanthine.

Particular spacer groups, e.g., at S1, S2, S3, W
20  and/or Y in the formula, are as described above. The
incorporation of spacer groups at S1 and S2 will affect
the relative distance between adjacent bases on NuAA
monomers. Preferably, the distance between adjacent
bases is at least about 4 carbon-carbon bond lengths,
25  more preferably at least about 5, still more preferably
about 6. This spacing can be achieved by including a
corresponding number of atoms between the E position of
one NuAA monomer and the E position of the next NuAA
monomer. Thus, to incorporate about 6 bond lengths, for
30  example, one can include 5 atoms along that length of the
backbone. The desired "spacer" atoms can be incorporated
within NuAA monomers (for example, by using monomers with
a propylene moiety at S2 or S1, or e.g. a methylene at S1
and an ethylene at S2). Most preferably, however, S1 is
35  a bond since that will facilitate the synthesis of NuAA
monomers from naturally occurring amino acids and their
derivatives. As described below there are a number of

20

commercially available amino acids that can be used as precursors for the synthesis of NuAA monomers having a variety of spacer groups at S2. As described in Example 2, molecular modelling can be used to assess the likely
5  impact of, for example, lengthening the spacer group at S2. Preferably, S2 is a methylene group, more preferably an ethylene group, still more preferably a propylene group.

Alternatively, the spacer atoms can be provided by
10 monomers located between NuAA monomers. Since aminoacyl monomers derived from natural amino acids will provide a three-atom backbone, these can be conveniently used between adjacent NuAA monomers. Such spacer AA monomers can also be used to provide D- or L-chiral centers.
15 Similarly, aza-AA monomers can be used to provide quasi-chiral centers between adjacent NuAA monomers. Thus, individual PENAMs can include one or more types of monomers and/or chiral centers.

The spacer group at S3 is used to affect the
20 distance between the nucleic base and the peptidic backbone. Molecular modelling has been used to show that, in order to mimic the homomorphism of native nucleic acids, the nucleic base is preferably substituted on the side chain at least two carbon units distance from
25 the peptidic backbone (e.g., $\gamma$-substituted), and is preferably not more than four carbon units away from the backbone. Such NuAA monomers can be easily synthesized from commercially available starting materials, for example, natural alpha-amino acids, as illustrated below.
30 The atom at E will generally be a carbon, in which case E can be a chiral center analogous to the alpha carbon of a natural amino acid (other than glycine). When E is a chiral center, then the NuAA monomers can be L- or D-NuAA monomers depending on whether the
35 configuration of groups around the chiral center is analogous to that found in an L- or D-amino acid (where the base segment is in the position normally occupied by

21

an amino acid "R" group and the carbonyl and amide groups are in the directions shown in the formula). As described herein, the PENAMs of the present invention contain D-chiral and/or quasi-chiral centers.

5      If E is carbon, then W and Y can be hydrogen or a spacer group as defined above. Most preferably, W and Y are hydrogen atoms.

When the atom at E is replaced isosterically with trivalent nitrogen in place of carbon, the resulting NuAA monomer (termed an "aza-NuAA monomer") can adopt configurations between D- and L- amino acids. Thus, there will generally be a lone pair of electrons at position Y; and, since the electrons are not fixed, the resulting center around E can adopt a variety of spatial configurations and is referred to as a quasi-chiral center.

NuAA monomers can be conveniently synthesized from generally available starting materials such as natural amino acids, unnatural amino acids and the derivatives thereof, see, e.g. [63-65]. As will be appreciated, a variety of possible synthetic routes can be employed. "Retrosynthesis" techniques can be used to identify particularly convenient pathways; some of which are illustrated below.

25     As described below, the design of such mimics can be tested by well-known molecular modelling techniques in order to assess the likely impact of a particular group on binding of the targeting bases of the side chain to their nucleic acid target.

30

2.    Assembly of Particular PENAMs from NuAA Monomers

The PENAMs of the present invention contain strings of NuAA monomers (although they may also contain other groups, as described herein). The sequence of bases in the PENAM can be made to complement any target nucleic acid sequence by simply incorporating the corresponding NuAA monomers into the PENAM. Thus, where a gene or

22

other nucleic acid target has already been at least
partially sequenced, then PENAMs can be designed to
interact with such a target nucleic acid according to the
standard "rules" of Watson-Crick base-pairing (i.e., a
5   single-stranded nucleic acid comprising a nucleotide
sequence CCGGTTAA could be targeted by a PENAM comprising
a base sequence GGCCAATT; note that longer sequences are
generally used, as described below).

Several basic considerations guide selection of a
10  suitable number of bases to include.  Two particularly
important consequences of increasing the length of the
complementary region are an increase in specificity and
an increase in affinity.   The increased specificity
derives from the fact that the general probability of
15  occurrence of a particular target sequence will be $(1/4)^n$,
where n is the number of bases in the target sequence.
Thus, the probability (P) that a particular sequence of
n bases will occur in a nucleic acid containing a total
of N bases can be estimated from the following formula:

20                              $$P = N \; / \; 4^n$$

For example, the probability that a 10 base sequence
will occur in a nucleic acid of 30,000 nucleotides (30
kb) would be estimated to be $3 \times 10^4$ / $4^{10}$ or about 0.03; and
the probability of a 15 base sequence occurring would be
25  approximately 0.0000279.   Thus, for targeting sequences
in a viral genome, most of which have less than 300 kb of
unique-sequence nucleic acid, a sequence of less than 15
bases, and perhaps as few as 9 or so, may provide more
than sufficient specificity of targeting.    In more
30  complex genomes, it is preferable to have a longer
sequence.   Thus, a 17 base sequence would be expected to
occur once in the entire human genome [8].

It is also the case that overall binding affinity is
improved by providing more bases for hydrogen bonding.
35  This explains the correlation observed in antisense ODNs
between chain length and antisense activity.    For
duplexes in the range of 10 to 20 base pairs (bp), the

23

melting temperature increases by roughly three degrees
Celsius for every additional base pair formed by two
hydrogen bonds (e.g., A:T) and by roughly six degrees
Celsius for every additional base pair formed by three
5  hydrogen bonds (e.g., G:C). Thus, a sequence number
selected on the basis of specificity [vis-a-vis the
complexity of nucleic acid in the targeted genome(s)] may
be extended to influence the affinity of binding.
However, the significance of the correlation between
10  length and affinity is greatly reduced once the number of
bases exceeds about thirty [8]. Furthermore, longer
sequences may be more difficult to synthesize and may
penetrate cellular membranes less efficiently. Of
course, a preferred approach will be to identify an
15  appropriate range, typically 10 to 25 bases, and then to
compare a set of oligomers of differing lengths within
that range to determine an optimum length. Synthesizing
a range of PENAMs of differing lengths and/or
specificities can be easily accomplished using the
20  present invention since the PENAMs are designed to be
prepared using modular synthesis on a peptide
synthesizer.

The particular bases incorporated within the PENAM
will of course depend on the desired nucleic acid target.
25  As is well known to those of skill in the arts of DNA
probes and antisense technology, the overall binding of
the targeting molecule to its target depends, inter alia,
on the length of the complementary sequences and the
number of mismatches within those sequences. Preferably,
30  the PENAMs of the present invention will be at least
"partially complementary" (60% homologous) to a stretch
of bases in the target nucleic acid. More preferably,
the PENAM will be "substantially complementary" (at least
about 80% homologous); most preferably, the PENAM will be
35  "completely complementary" (100% homologous). As
discussed above, however, it is quite feasible to
incorporate special base analogs (such as inosine) to

24

effectively broaden the binding capability for one or more positions in the monomer (if, for example, it represents a position that is unidentified, or is likely to vary in a given population of target sequences). It

5  is also feasible to incorporate other analogs to further enhance binding; see the illustrations above regarding the use of modified bases with enhanced affinity, and the use of cross-linking moieties.

     PENAMs can also target double-stranded target

10  nucleic acids via triple helix formation, as discussed below.

     It is also possible to combine the PENAM technology of the present invention with the use of standard oligo(deoxy)nucleotides (i.e., "ODNs"). Thus, for

15  example, a PENAM might include a sequence of "natural" nucleotides. However, since the predominant activity of exonucleases appears to be 3' to 5' it would be preferable to have at least the 3' end of such an ODN linked to another portion of the PENAM.

20

Homomorphism

     The PENAMs of the present invention have a peptidic backbone that incorporates unusual chiral centers

25  (including D-chiral centers and/or quasi-chiral centers) that can be used to orient the nucleic side chains in such a way that the nucleotidic bases are spatially homomorphic to bases in targeted nucleic acids. The ability to enhance binding by spatial homomorphism is

30  especially significant given that hydrogen bonding interactions between biomolecules typically depend on an aggregation of many relatively weak bonds.

     For optimal binding, it is believed that the PENAM should have a conformation in which the nucleotidic bases

35  are stacked approximately at the 3.5 Angstrom interval and are oriented away from the backbone in such a manner that the hydrogen bond donors and acceptors of the

25

nucleotidic bases can directly "read" the complementary
bases on the target nucleic acids by specific hydrogen
bonding interactions.  That is, the PENAM and the target
should be substantially homomorphic.  In order to achieve
5    such homomorphism, the PENAMs of the present invention
comprise strings of NuAA monomers (and may comprise other
intervening monomers) in which the NuAA monomers (or the
intervening monomers, or both) can incorporate unusual
chiral centers, including D-chiral centers and/or quasi-
10   chiral centers.

    The various monomers and chiral centers can be
incorporated into PENAMs in a variety of different
configurations.    Thus,  PENAMs  can  incorporate,  for
example, mixtures of L- and D-chiral centers; which can
15   be   in   unequal   proportions   or   in   roughly   equal
proportions.  PENAMs with roughly equal proportions of L-
and D-chiral centers can have these centers dispersed
(e.g. wherein at least about 85% or L-chiral centers are
flanked by D-chiral centers).  Higher proportions of D-
20   chiral centers can also be used (e.g. wherein at least
about 80% of the centers are D-chiral centers).  Quasi-
chiral centers can be incorporated in addition to, or in
place of, D- and/or L-chiral centers; and these allow the
resulting   PENAM   to   adopt   a   variety   of   spatial
25   conformations  at  the  quasi-chiral  centers.    Molecular
modelling with energy minimization of PENAM strands in
association  with  a  target  DNA  strand,  as  illustrated
below, can be used to predict the preferred positioning
of the particular chiral centers and/or quasi-chiral
30   centers for a particular PENAM structure.

    NuAA monomers can be joined directly to each other
or they can be separated by intervening monomers of other
types.   Preferably,  such  other  monomers  (or  strings
thereof) are amino acids and/or aza-amino acids, or their
35   derivatives,  since  all  of  these  monomers  can  then  be
conveniently   polymerized   via   peptide   synthesis
techniques.   Of course,  it will also be possible to

26

separately prepare larger fragments, such as polypeptide fragments, and then incorporate such fragments into a PENAM.

As discussed herein, the peptide backbone of the

5    PENAMs of the present invention serves as a scaffold to orient the side chains (-S3-B in the formulae) in such a way that the nucleic bases substituted in the side chain can "read" the complementary nucleic bases sequence on the target nucleic acid. From the rational drug design

10   point of view, the peptide backbone scaffold can be c h e m i c a l l y    m o d i f i e d    t o    a l t e r    i t s hydrophobicity/hydrophilicity, or its electronic and/or stereochemical properties [66-68]. An exemplary but not exhaustive list of such chemical modifications includes:

15   1) modifications of the amide nitrogen; 2) modifications of the alpha-carbon (where E is carbon); 3) modifications of the amide carbonyl; and 4) modifications of the amide bond. Decreasing the hydrophilicity of the backbone can be expected to improve uptake of the molecules.

20   Decreasing the electronegativity of the backbone can also be expected to enhance binding to a complementary nucleic acid (which is itself electronegative). In these regards, the PENAMs of the present invention (in which the polyphosphate-sugar backbone has been entirely

25   replaced) are already substantially improved relative to standard ODNs. However, additional modifications affecting hydrophilicity/electronegativity can also be incorporated. As will be apparent to those of skill in the art, the incorporation of cationic or anionic amino

30   acids as spacer AA monomers would render the PENAM cationic or anionic, respectively. Thus, "basic" amino acids such as lysine or arginine can be conveniently used to render the PENAM cationic in nature, and "acidic" amino acids such as aspartate or glutamate can be

35   conveniently used to render the PENAM anionic in nature. Making the PENAM cationic would of course be expected to electrostatically enhance the interaction between the

PENAM (cationic) and the phosphate backbone of DNA/RNA (anionic). Modifications in the backbone (especially at S2 and/or S3) can be used to favor particular conformations (e.g. helix vs. beta-sheet) and to thereby

5    improve base stacking.

Molecular modelling techniques can be used to assess the likely impact of a particular group (such as a larger spacer group) on binding of the targeting bases of the side chain to their nucleic acid target. A number of

10   suitable molecular modelling techniques are known in the art [69-75], and computer software incorporating such techniques is also available, as described below.

Molecular modelling, often synonymous with computer-aided molecular design, is a general methodology

15   which comprises several computer-based methods for drug design [76, 77]. Using these methodologies, one can readily assess the impact of introducing various modifications into targeting molecules such as the PENAMs of the present invention. A number of publicly available

20   modelling packages, including BIOGRAPH-II*, CHEMLAB-II*, CHEM-X*, HYPERCHEM*, MACRO MODEL*, SYBYL*, and others, can offer some or all of the following advantages that can be used to design and check particular PENAMs of the present invention. Some of the useful features of these systems

25   are outlined below.

Model building and computer graphics programs allow users to "build" a molecule from scratch or import it from 3D structure data bases and also allow manipulations of the displayed model, ranging in size from small

30   molecules to macromolecules in 3D. They also enable the user to dock more than one ligand into the putative active site of the macromolecule and to manipulate the display simultaneously or individually. Publicly available programs of this type include, for example:

35   ALCHEMY*, from Tripos associates; MIDAS PLUS*, from The University of California, San Francisco; FRODO*, from Rice University, Houston; HYDRA* from Polygen; MANOSK* from

28

University of Paris; MOLECULAR GRAPHICS° from ACS software; and MOGLI° from Evans and Sutherland. Molecular model building programs like CONCORD° and COBRA° can also be used to generate low energy 3D structures from 2D on
5    work stations [78].

Molecular mechanics and dynamic simulation programs allow model refinement by optimizing the rough coordinates of the model, utilizing either quantum mechanics or molecular mechanics calculations. Molecular
10   mechanics can optimize the 3D structure of a molecule by moving to the nearest local minimum energy structure. Even though both quantum mechanics and molecular mechanics have their basic differences in calculating the potential energy function of an isolated molecule or a
15   biological system, they complement each other and can be used to optimize the interaction energy and the binding conformation between the ligand and its target macromolecule [79, 80]. Molecular dynamics simulations can be used to follow the solvation and conformational
20   changes involved in the interactions between the ligand and its receptor on initial binding, and also any conformational or covalent rearrangements which may occur on subsequent binding. Molecular dynamics can be used to assess entropies, enthalpies and other thermodynamic
25   quantities for various molecular configurations [81]. Accordingly, these calculations can be used to predict how variations in the basic structure of a PENAM will affect the equilibrium constant for binding to its target.    Publicly available programs of this type
30   include, for example: MM2° and MOPAC°, from Quantum Chemistry Program Exchange; and MMP2°, from Molecular Design Ltd.    In addition, AMBER° and GROMOS°, from The University of California, San Francisco, are two programs available for molecular mechanics and dynamics
35   simulations [80].

Docking, scoring and screening algorithms can also be used as computer screening procedures to test lead

29

candidates for binding to putative ligands. The docking
criteria typically involve either geometric orientation
or steric match and evaluating the goodness of fit of the
"ligand" to the active site. The scoring algorithms use
5    steric complementarity, full intermolecular force field
and other molecular properties as the scoring functions.
Publicly available programs of this type include, for
example: DOCK*, from The University of California, San
Francisco [82-84]; GEOM*, from the Cambridge Structural
10   Data Center; ALADDIN*, from Daylight chemical Information
Systems, and MACCS-3D* from Molecular Design Ltd. [78].

Quantitative structure-activity relationships (QSAR)
and statistical programs can be used to correlate the
biological potency of a series of analogs with the
15   relative values of physical properties such as partition
coefficients (log P), $pK_a$ values, and electronic or steric
properties and the size of the substituent. In addition
to QSAR, the comparative molecular field analysis
(CoMFA), which is a combination of receptor mapping,
20   potential energy calculations and QSAR, uses the relative
potency of molecules and their superimposed bioactive
conformations to calculate steric, electrostatic and
hydrogen-bonding interaction energies for each molecule
[85, 86]. Statistical analysis of these energy values
25   with respect to their biological potency or affinity can
be used to predict the affinity of the proposed molecule
for a given target. Publicly available programs of this
type include, for example: MEDCHEM*, a modelling and
information management program developed by Corwin Hansch
30   at Pomona college [87], which includes algorithms to
calculate log P values; and ADAPT*, another complete
program, developed by Jurs et al. [88] and marketed by
Molecular Design Ltd., which performs all the operations,
i.e., vector representation of the molecules, statistical
35   analysis by various methods, data input and management.

As an illustration of these techniques, Example 1
describes the use of molecular modelling to analyze the

30

stereochemical impact of changes to the chirality of the peptidic backbone in the PENAMs of the present invention. In particular, a model of the PENAMs of the present invention was prepared in which the peptide backbone 5 comprises alternating chiral centers, and this was compared to a model in which the backbone was assembled from "natural" L-amino acid-like monomers. As described in the example, the modelling data indicate that the ability to incorporate centers of varying chirality 10 substantially improves the homomorphism or "fit" of the PENAM to its target nucleic acid. As described herein, centers of varying chirality including D-chiral, L-chiral and quasi-chiral centers can also be introduced into spacer monomers such as amino acids, incorporated between 15 adjacent NuAA monomers. Of course, the inclusion of small achiral amino acids such as glycine as spacers would be expected to have the least effect on the overall configuration of the PENAM; whereas the inclusion of L- and/or D-amino acids can be used to alter the 20 conformation of the PENAM.

It is known that DNA and RNA can exist in several secondary structures or isoforms such as right-handed A- and B-forms, as well as the left-handed Z-form, and it is believed that these isoforms could be essential for 25 specific biological functions [89]. These polymorphisms in the double helix arise from differences in base stacking, backbone geometry, and the sugar conformation of the particular form. Each of these forms differ distinctly from each other with respect to helical 30 hardness, as well as the shape and size of the helical groove. These distinct features have been effectively exploited to design conformation-specific metal complexes by Barton et al. [90]. Like synthetic ODNs, the PENAMs of the present invention are expected to be capable of 35 binding to each of the various isoforms of the nucleic acids [91-93]. The molecular modelling techniques described above can be effectively used as a selection

31

tool to assess the "homomorphic fit" of a particular
PENAM to a generated molecular model of a specific
nucleic acid isoform.

5    Assembly of PENAMs from NuAA Monomers

Since the NuAA monomers can be joined via peptide
bonds, the PENAMs of the present invention can be
conveniently assembled using well-known peptide synthetic
techniques.   These building block NuAA monomers thus
10   allow for the rapid production of a variety of
"antisense" oligomers, exhibiting differing sequences for
targeting complementary nucleic acids, via an automated
peptide synthesizer.

Solid phase peptide synthesis (SPPS) is a convenient
15   and powerful methodology for the synthesis of a wide
variety of oligopeptides in large quantity and high
purity [94].  The methodology involves three basic steps:
(i) peptide chain assembly; (ii) peptide cleavage, side
chain group(s) deprotection and isolation from polymeric
20   support; and (iii) purification and characterization of
the oligopeptide.

The two most common synthesis strategies for SPPS
are based on the deprotection chemistry of either t-Boc
or Fmoc groups, used for the protection of the amino ($NH_2$)
25   group of the amino acid.   The choice of t-Boc or Fmoc
depends upon the protection and deprotection chemistry of
the functional groups of each monomer.

The first step in chain assembly is to attach the
$\alpha$-carboxyl group of the first t-Boc- or Fmoc-protected
30   amino acid or aminoacyl monomer through a linker to a
solid polymeric support.  The next step is deprotection
or removal of the t-Boc or Fmoc protective group of the
first amino acid.  The t-Boc protecting group is removed
by an acid, usually trifluoroacetic acid (TFA), whereas
35   Fmoc group is deprotected by base, usually piperidine.
After deprotection, the next amino acid is coupled to the
deprotected amino (NH2) terminus through its $\alpha$-carboxyl

32

(-COOH) group to form a peptide bond. Through successive cycles of deprotection and coupling, the growing peptide is assembled from the C-terminal towards the N-terminal to a desired chain length. Once the desired chain length is achieved, the oligopeptide is cleaved from the polymeric support either by a strong or weak acid. Also, depending upon the type of the resin linkage on the polymeric support, the cleaved oligopeptide has free carboxyl or carboxyamide at the C-terminus.

As described herein, the PENAMs of the present invention are unlikely to be degraded by either cellular nucleases or peptidases because of their unique structure which no longer resembles either nucleic acids or natural peptides. In addition, the uncharged backbone of PENAMs facilitates transport across the cell membrane. Uptake and transport across cellular membranes can be further enhanced by conjugating the PENAMs with carrier molecules at the N-terminus, at the C-terminus, or at an internal position in the PENAM. Carrier molecules include small reactive chemical groups (e.g., fluorescent dyes, intercalators, cross linking agents, alkylating agents, chain cleaving agents, biotin, digoxigenin, cholesterol, etc.) or stretches of other peptide-based compounds, including natural, modified or synthetic oligopeptides, or proteins (e.g., peroxidases, IgG, alkaline phosphatases and nucleases). They can be readily joined to the PENAMs via peptide bonds either during the synthesis or at the end of the synthesis on the peptide synthesizer [9]. Various strategies known in the art can be used to facilitate the uptake and selective subcellular localization of the PENAMs [95-109]. These strategies include:

1) increasing hydrophobicity by chemical modifications (for example, insertion of small chemical groups such as methylene, ethylene, propylene at S1 or, more preferably at S2 in a NuAA monomer) or by appending lipophilic

33

groups such as cholesteryl and its derivatives and/or other long chain fatty triglyceryl residues;

2)    physical or chemical association with polycations (for example, biotinylated molecules complexed to avidin can also exhibit increased cellular uptake, most probably due to the increased binding to the negatively charged cell surface (polycations would also enhance the binding of the PENAM to negatively-charged DNA);

3)    conjugation to ligands recognizing membrane proteins and receptors (for example conjugation to mannose-6-phosphate substituted serum albumin or mannosylated streptavidin); of course, conjugation to ligands that associate with cell-specific receptors (e.g. conjugation to a cytokine that binds only to cells bearing a cognate cytokine receptor) can be used to specifically "target" the PENAM to a desired sub-population of cells;

4)    delivery through nanoparticles made up of biodegradable polymers, such as polycyanoacrylate;

5)    poly(L-Lysine) mediated delivery; and

6)    association with lipoprotein or liposomes.


## Tandem Targeting

A modification of the approach that may be especially effective is the use of "tandem targeting" in which more than one different oligomer is employed as described by Maher and Dolnick, and Goodchild et al. [110, 111]. In particular, the use of two or more distinct targeting sequences (complementary to adjacent regions of the target) may result in synergistic effects on the modulation of the target. Preferably, the gap between the adjacent regions of complementarity will be less than about 15 bases, still more preferably the gap will be less than about 2 bases, and most preferably there will be no gap.

## Triple Helix Formation

Another way of targeting double-stranded DNA is via triple helix formation. There are several different motifs which are known to facilitate triple helix formation. In the "CT" motif, G-C basepairs are recognized by C residues and A-T basepairs are recognized by T residues - resulting in T-A-T and C-G-C* triplets across the three strands of the triple helical structure. This system is effective where one strand of the double-stranded target sequence contains only, or mostly, purine bases. An exception which may allow for a number of pyrimidine residues in the target sequence has been described by Griffin et al. [112]. The pyrimidine-rich targeting oligomer associates with the duplex such that the polarity of the oligomer is parallel to that of the strand containing the purine-rich target sequence. In situations in which a shorter purine-rich stretch is adjacent to a pyrimidine stretch, the oligomers may be designed to possess corresponding regions of inverted polarity such that the oligomer binds to the purine-rich stretch of one strand and then binds to the adjacent purine-rich stretch of the opposite strand. The recognition rules of the CT motif are further described by, for example, Maher et al. [113]; and Moser et al. [114]. One problem that may affect the use of the CT system is the ionization state of the targeting C residue at physiological pH. In order to facilitate the appropriate hydrogen bonding, the amino group at position 3 of the C must be protonated. This presents no problem when the pH is low; however, at neutral pH, most of the pyrimidines are unprotonated. If the CT system is to be used at physiological pH, it may be preferable to employ 5-methylcytosine or 5-bromouracil in place of cytosine; as described for example by Povsic et al. [115]; and Lee et al. [116]. It may also be especially useful to enhance stability of the triple helix by employing a

PENAM which comprises a group which will cross-link or intercalate into the target double helix.

In triple helix formation via the "GT" motif, guanine residues recognize G-C pairs, and thymine or
5   adenine residues recognize A-T pairs, as described for example by Cooney et al. [117]. In the GT motif, the targeting oligomer is in an anti-parallel orientation vis-a-vis the target sequence. Thus, via one or another sort of triple helix formation, a PENAM can be used to
10  target a complementary sequence on one strand of a double-stranded target [118-137]. The efficiency of Hoogsteen binding can be further enhanced by a number of well-known techniques. For example, as discussed above, 5-methylcytosine or 5-bromouracil in the targeting
15  sequence can be used to enhance binding to complementary G residues in the target sequence. Other approaches to alter the biologic effects of triple helix formation include the introduction of modifying groups onto the targeting nucleic acid or PENAM, such as intercalating or
20  crosslinking groups. For example, an acridine derivative can be introduced onto an end of a targeting sequence to enhance triple helix stability. These and other modifications are further described in Matteucci and Bischofberger and citations therein [2].
25

Variations of Basic PENAM to Modify Target

As discussed herein, a variety of functional groups can be introduced into the basic PENAM structure in order to further affect the interaction between the PENAM and
30  its target nucleic acid, and/or to affect the target nucleic acid itself. These reactive groups can be appended either at the N-terminus or at the C-terminus or at an intervening position. Examples of such reactive groups, discussed herein and in the art include labeling
35  groups, intercalating groups, cleaving groups and other groups that reconform or bind to nucleic acids or modify the target nucleic acid [3, 9].

36

One type of modifying group that can be introduced into a PENAM is a nucleic acid intercalating group. A number of such intercalating groups are known in the art, many of which are acridine derivatives [138-148].

5      Another type of modifying group is a cross linking group. Cross linking can be used to stabilize the interaction between a PENAM and its target, which may be especially useful in achieving and stabilizing triple helix formation. Various approaches to the stabilization

10     of triple helix formation include photochemical crosslinking as described by Le Doan et al. [149] and Praseuth et al. [150], and alkylation of the N7 of specific guanines in the target duplex as described by Vlassov [151] and Fedorova et al. [152].

15     Crosslinking can also be used to covalently link a new molecular structure, attached to the PENAM, to a particular location within a nucleic acid target. Thus, for example, a label attached to a PENAM could be linked to the particular location targeted by the PENAM. Such

20     labels could be photo-induced cross-linking agents, for example psoralen, coumarin, ellipticine and their derivatives [153-160].

Other labels might not involve a cross-linking group. A number of such labeling groups are known in the

25     art [161-166]. The ability to label particular sequences in a nucleic acid will also be useful in efforts to map and sequence various genomes.

Another type of modifying group that can be introduced into the PENAMs of the present invention are

30     nucleic acid alkylating agents. A number of such alkylating groups are known in the art. For example these include N-mustards as reactive alkylating compounds [167-177], porphyrins [161, 178], psoralens as photochemical activatable agents [158, 179] and quinones

35     as inducible alkylating agents [180].

Another type of modifying group that can be introduced into the PENAMs of the present invention are

37

nucleic acid cleaving groups.  There are a number of
cleaving groups that can be used to allow a PENAM to act
as an artificial sequence-specific nuclease, which have
been described in the art [125, 181-192].  The following
5  approaches are intended as an illustrative, not an
exhaustive, list of cleaving groups.  In one approach,
iron(III) EDTA is used as a cleaving group which
generates   free   radicals   under   appropriate   redox
conditions as described by Moser et al. [114].  Other
10  redox-activated transition metal cleaving groups include
complexes   of   o-phenanthroline-Cu(I)   introduced   by
Francois et al. [193, 194] and porphyrins-Fe(II) used by
Le Doan et al. [195].  These systems may be more useful
in  vitro,  where  redox  activation  is  more  readily
15  controlled.    Another   alternative   is   photochemical
cleavage as described by Perrouault et al. [154].  Still
another approach is to incorporate as a cleaving group a
relatively  non-specific  nuclease  such  as  DNase  I  or
staphylococcal nuclease and effectively convert it into
20  a specific endonuclease by conjugation to the sequence-
specific PENAM, analogous to the work reported by Schultz
et al. involving ODNs [196-198].  Yet another possible
approach   to   cleaving   target   nucleic   acids   is   to
incorporate a ribozyme into the PENAM [199-201].
25     Depending   on   the   particular   modifying   groups
selected, these groups can be incorporated into the PENAM
at virtually any position, including positions within and
outside of the NuAA monomers.  However, there are a
number   of   general   considerations   that   should   guide
30  selection of a particular group and location.  The most
significant consideration is that the group should not be
introduced into a position that is likely to prevent
sufficient hydrogen bonding between the bases of the
PENAM and those of the target nucleic acid.  Thus, while
35  small modifying groups can be accommodated within the
NuAA monomers engaging in hydrogen bonding to the target,
larger groups may be better accommodated outside of the

hydrogen bonding monomers. Even large modifying groups such as nuclease enzymes can be attached to such terminal regions of the PENAMs [8]. In some cases, the nature of the interaction between the modifying group will dictate
5 favorable positions in the PENAM. Moreover, molecular modelling can be used to anticipate favorable positions for the incorporation of such groups.

The binding affinity of candidate PENAMs can be readily tested using any of a variety of well-known
10 techniques. These include, for example, standard melting temperature ($T_m$) measurements [202-204]; as well as chemical, photochemical, physical and/or enzymatic probing [205-213]. Binding can also be effectively probed using "footprinting" techniques. Footprinting was
15 primarily introduced by Galas and Schmitz [214] to detect contacts between DNA and DNA binding proteins. The basic idea behind this methodology is to allow a protein to bind to radioactively-labelled DNA/RNA containing the sequence that is recognized by the protein. After the
20 binding event, the protein-DNA complex is subjected to nuclease digestion, usually using DNAse I. The regions of the DNA molecule covered by the protein are protected from the digestion while the rest of the DNA backbone is digested. The products of this cleavage produce blank
25 regions or "footprints" at the sequence where the protein was bound to the DNA sequence (as visualized on autoradiographs of electrophoretic gels such as those used for DNA sequencing). Thus, by comparision with a DNA sequence marker, one can identify the sequence
30 specificity of a particular DNA binding moiety. Various chemical reagents have been developed to enhance fingerprinting analyses. Among them, reagent systems consisting of [Fe(EDTA)$^{-2}$], hydrogen peroxide and sodium ascorbate [215-217] and gamma rays [218] are noteworthy.
35 Numerous examples of this technique to study the sequence-specificity of nucleic acids by oligonucleotides are known in the art [128, 148, 219-221].

39

## UTILITY AND ADMINISTRATION

### Diagnostic/Analytical/Preparative Applications:

The peptide-based nucleic acid mimics (PENAMs) of
5    the present invention are substantially homomorphic with
nucleic acids.  This homomorphism facilitates hydrogen
bonding and thus hybridization with target nucleic acid
sequences.

Diagnostic, analytical and/or preparative uses of
10   PENAMs include the use of PENAMs as "reporter" molecules;
use of PENAMs for the capture of complementary DNA/RNA
sequences.  For example, the PENAMs can be used as
hybridization probes to identify target sequences in a
particular DNA or RNA of interest [222-233].

15

### Use as a Reporter

Generally, for diagnostic applications, the PENAMs
will comprise a labeling or "reporter" group (as
described herein) by which the interaction with the
20   proper target sequence can be detected [234-242].  A
typical example of such an application would involve a
pool of single-stranded polynucleotides (the sample or
"analyte" which might contain a target sequence such as
a viral nucleic acid).  In a typical probe approach, this
25   pool of polynucleotides will be bound to a solid support.
A labeled PENAM is then introduced to the polynucleotides
under hybridizing conditions and allowed to anneal.
After washing away non-hybridized PENAM, the sample bound
to the solid support is analyzed for the presence of the
30   labeling group.  Such assays can be carried out according
to standard hybridization procedures suitable for the
degree of complementarity expected.  That is, high
stringency hybridization can be used for high levels of
complementarity (e.g. greater than 90%), lower levels of
35   stringency for lower levels of hybridization, as is well-
known in the art.  However, given the reduced charge
repulsion between PENAMs and nucleic acids, and also the

40

possibility of introducing intercalating or cross-linking
groups into the PENAMs, it will be possible to design
PENAM probes that can be subjected to even more stringent
hybridization conditions (e.g., higher temperature, lower
5   salt) than the corresponding native complementary strand.
The ability to use more stringent conditions will reduce
the background "noise" caused by non-specific or
mismatched binding and, as a result, will reduce the
incidence of false-positive results.  For a given PENAM
10  and target, the optimum hybridization conditions can be
determined by testing the PENAM under varying conditions
(particularly the concentration of denaturants, the
temperature during hybridization and washing, and the
concentration of salts during washing) with both positive
15  and negative controls (known amounts of target sequence
decreasing to zero).

## Use for Capture

In another type of diagnostic/analytical/preparative
20  application, the PENAMs can be linked to a solid support
for capture of complementary (target) nucleic acids to
the support [243-245].  In such systems, the PENAMs can
also be synthesized directly onto the solid support.  The
presence of target nucleic acid bound to the solid
25  support via the PENAM can be detected, for example, by
using a polycationic molecule which would bind to the
negatively charged backbone of the target nucleic acid
(but not the peptidic backbone of the PENAM).   The
polycationic molecule can comprise a labeling group to
30  produce a signal by which the bound complex can be
detected.  The PENAMs of the present invention can also
be employed in the variety of sandwich hybridization
assays which are known in the art.  As discussed above,
one advantage of using PENAMs is the ability to run the
35  assays under more stringent hybridization conditions.

The use of PENAMs as capture probes can also be
applied to preparative techniques in which the PENAM,

41

typically bound to some support, is used to attract and thereby retrieve a complementary target nucleic acid using methods analogous to those described for the diagnostic assays above.

5

Therapeutic Applications:

Antisense and/or Antigene Modulation

The PENAMs of the present invention are also useful, as described herein, for modulating the activity of
10  nucleic acids in an antisense manner.  The ability to modulate nucleic acid activity by antisense regulation is well known in the art as described [5, 8, 121, 246-250]. With respect to the control of gene expression, PENAMs can be used not only to inhibit expression but also to
15  activate it in vitro as well as in vivo.  Indirect activation of gene expression can be accomplished, for example, by suppressing the biosynthesis of a natural repressor, as described for antisense ODNs by Inoue [251].  Direct activation of gene expression can be
20  accomplished, for example, by reducing termination of transcription as described for antisense ODNs by Winkler et al. [252].  There are several in vitro as well as in vivo test systems known in the art that have been routinely used [95-97, 101, 109, 253-265].  The efficacy
25  of PENAMs as antisense or antigene chemotherapeutics can be easily tested and compared using these test systems

The ultimate targets of antisense modulation include viral diseases, bacterial diseases, inborn errors of metabolism, malignant cell growth, and any other
30  conditions associated with the presence of a characteristic DNA or RNA or products thereof [7, 266-270].

Preparation/Administration
35       Depending on the nature and location of the target nucleic acid, there are a number of different techniques that can be used for promoting contact between a PENAM

42

and its target. For example, where the target nucleic
acid is in solution in vitro, the PENAM can simply be
added to the solution and the mixture incubated under
standard conditions favoring nucleic acid hybridization.
5  Where the target is intracellular, the PENAM must be
capable of passing through one or more cellular membrane
systems in order to contact the target nucleic. In this
regard, the PENAMs of the present invention are already
substantially more effective than nucleic acid based
10 targeting    molecules    such    as    antisense
oligodeoxynucleotides (ODNs). In particular, the passage
of ODNs through cellular membranes is very inefficient
because of their highly-charged backbone. In the case of
PENAMs, the bases are attached to a peptidic backbone
15 which is far less charged at physiological pH. If
desired, the permeability of PENAMs can be further
enhanced by incorporation of additional modifications.
A number of such modifications are known in the art and
have been described in detail earlier [2, 4]. These
20 modifications include, for example, the attachment of
hydrophobic chains to the targeting molecule [271, 272].
Another approach involves the attachment of polylysine
[105], however, polylysine may not be applicable in all
cell systems [4]. Another non-specific carrier which can
25 be used to improve uptake is cholesterol and its
derivatives [102, 273, 274].

Another approach is to utilize receptor-mediated
endocytosis to enhance and/or to direct cellular delivery
[2, 4]. In this approach, cells with receptors for a
30 particular ligand can be targeted by linking the ligand
to the PENAM. By way of example, linking biotin, folate,
transferrin or interleukin-1-$\beta$ (IL-1B) to a PENAM of
interest, one can specifically and efficiently target
cells expressing biotin, folate, transferrin or IL-1B
35 receptors. For situations in which the receptor status
of a cell type is unknown, or where a receptor-ligand
pair is known but the ligand cannot be suitably linked to

43

the PENAM, it may also be possible to use more generalized ligands such as lectins (sugar-binding proteins) since most cells are believed to have glycoproteins on their surface. Such a generalized
5  approach may also be useful where targeting a broad range of cellular types is desired. Analogous approaches can be used to target nucleic acids within a cellular organelle. For example, linking a mitochondrial protein to a PENAM can be used to target the PENAM to
10  mitochondria as has been described for incorporating double-stranded DNA into mitochondria [4]. Of course, such receptor-mediated delivery systems may utilize any of a large number of other known cellular receptor systems.

15     Still another approach is to deliver the PENAMs to target cells using liposomes [98] or antibody targeted "immunoliposomes" [2]. Linking of acridine or benzophenanthridine to the PENAM can be used to improve incorporation into neutral liposomes [4].

20     The PENAMs are thus useful in therapeutic, diagnostic and research contexts. PENAMs can be administered in the same manner as modified ODNs, which manner of application is conventional in the art. In therapeutic applications, the PENAMs are utilized in a
25  manner appropriate for antisense therapy in general. For such therapy, the PENAMs can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations may be found, for example, in Remington's
30  Pharmaceutical Sciences, Mack Publishing Co., Easton, PA. (latest edition). The PENAM active ingredient is generally combined with a carrier such as a diluent or excipient which may include, for example, fillers, extenders, wetting agents, disintegrants, surface-active
35  agents, or lubricants, depending on the nature of the mode of administration and the dosage forms. Typical dosage forms include tablets, powders, granules,

44

capsules, suppositories, liquid preparations such as suspensions, emulsions and solutions, and liquid preparations for injections, including liposome preparations. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the PENAMs of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the PENAMs may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example. For oral administration, the PENAMs are formulated into conventional oral administration forms such as tablets, capsules and tonics. For topical administration, the PENAMs of the invention are formulated into ointments, salves, gels or creams, as is generally known in the art.

The PENAMs of the present invention will, by virtue of their unusual structural features, be resistant to the degradative enzymes that are expected to be present in most biological systems. Thus, the PENAMs do not possess the phosphodiester backbone which is the standard target of the nucleases. Also, the peptidic backbone is unlike that of naturally-occurring peptides because of the presence of both D- and L- residues. This basic resistance to degradative enzymes can be further enhanced, if desired, by the addition of protecting

45

groups which interfere with the ability of a degradative
enzyme to modify the oligomer.


<u>Examples</u>

5       The following examples are intended to illustrate,
not to limit, the invention.


<u>General procedures</u>

        Unless otherwise noted, the following general
10  procedures were employed in conducting the experiments
subsequently described:

        All moisture-sensitive chemical reactions, requiring
inert atmosphere, were carried out under nitrogen or
argon in oven- or flame-dried glassware using septum
15  technique.  All chemicals and solvents were of technical
or reagent grade and used as received unless otherwise
stated.  Thin layer chromatography (tlc) was carried out
on pre-coated 0.25 mm layer thickness silica gel and/or
alumina plates, supplied by Analtech Co.  Preparative
20  thin layer plates were coated with 1.5 mm layer thickness
silica gel GF-254 (60 mesh, ASTM).  Flash column
chromatography was carried out using 70-230 mesh silica
gel, supplied by E. Merck.  Melting points were taken on
an  electrothermal  apparatus  and  are  uncorrected.
25  Infrared  spectra  were  recorded  on  a  Nicolet  FT-IR
spectrophotometer  with  samples  prepared  as  potassium
bromide  pellets  or  as  thin  films  on  NaCl  plates.
Ultraviolet spectra were recorded in quartz cuvettes on
Hewlett Packard UV/VIS spectrophotometer.  NMR spectra
30  were recorded on GE 300 MHz or GE 500 MHz spectrometers
using  deuterated  solvents  and  tetramethylsilane  as  an
internal  standard.  Mass spectra were recorded at UCSF
Mass  Spectrometry  center.  Optical  rotations  were
measured in a 1-dm quartz cell at 22-23 degrees Celsius
35  at the sodium D line on a Perkin-Elmer polarimeter (Model
241 MC).  Solid Phase Peptide Synthesis was carried out
on  an  Applied  Biosystems  Peptide  synthesizer  (model

46

431A), using Boc-strategy. Hydrogen fluoride setup was used to cleave the peptides from the resin. The peptides were purified either by HPLC using C18 reverse phase Vydac® column or by FPLC using cation exchange Mono S° column.

## Example 1
### Basic molecular comparison of PENAMs of the present invention with standard ODNs and with PENAMs that do not contain centers of varying chirality

As shown in Figure 1, the chimeric PENAMs of the present invention are structurally quite distinct from standard oligonucleotides (ODNs). It is by virtue of these differences that PENAMs avoid a number of technical difficulties associated with the transport and stability of ODNs. Functionally, however, the PENAMs are analogous to ODNs in their ability to engage in sequence-specific hydrogen bonding to complementary nucleic acids by virtue of their nucleotidic sidechains being properly "presented" by the peptidic backbone. Molecular modelling has been used to analyze and confirm these structural and conformational relationships.

Design and model building computations were performed on a Silicon Graphics (Mountain View, CA) IRIS work-station [275] using the SYBYL° molecular modelling package (v.5.4/6.0), offered by Tripos Associates [276]. There are several ways to generate or build these models. As an illustration, the following approach has been used to build comparative models

Firstly, to illustrate the substantial structural differences between the PENAMs of the present invention and standard ODNs, molecular modelling was used to show the steps required to "convert" DNA into a PENAM. The SYBYL DNA sequence builder was used to generate the complementary double stranded DNA decamer, $dT_{10} \bullet dA_{10}$, into B-DNA conformation, based on the crystal structure, solved by Arnott et al. [277]. The purine strand ($dA_{10}$)

47

of the generated DNA decamer, $dT_{10} \bullet dA_{10}$ was then used as a
template to construct examples of the PENAMs: Version I
(without varying chiral centers) and Version II (with
alternating chiral centers).

5      First, the oxygen atoms (O4') of the ribose rings,
along with the C4'--O4' and O4'--C1' bonds, were deleted.
Then, all of the O3' oxygen atoms and C4' carbon atoms
were substituted by carbonyl (C=O) groups and amide
nitrogens (N), respectively. Next, the C5' carbons, the
10 O5' oxygens and the remaining phosphate backbone (⁻O-P=O)
were deleted. Next, trans-amide bonds were made between
the amide nitrogen (N4') of each monomer and the carbonyl
(C=O3') of the next monomer. Appropriate hydrogen atoms
were added on all of the modified atoms (C1', N4') to
15 satisfy their hybridization states. Having effectively
transformed the sugar-phosphate backbone into a peptidic
backbone, the side chains were then substituted with the
nucleic bases (adenine in this illustration), to generate
a chimeric peptide-based nucleic acid mimic.

20     In order to compare the homomorphism of PENAMs with
and without varying chiral centers, two different
versions of the previously described PENAM were
constructed.   In PENAM Version I (without varying
chirality), all of the alpha carbons (C2) were assigned
25 the L-configuration as in the "natural" L-amino acids.
PENAM Version I was then energy minimized as a single
strand, in beta-sheet-like extended conformation, using
force field parameters offered by SYBYL software package.

        In PENAM Version II, all alpha carbons (C2) of odd
30 numbered residues were assigned the L-configuration as in
"natural" amino acids, and all alpha carbons (C2) of even
numbered residues were assigned the D-configuration as in
"unnatural" D-amino acids.   PENAM Version II was then
energy minimized as a single strand, in beta-sheet-like
35 extended conformation, using force field parameters
offered by the SYBYL software package.

48

As discussed above, it is believed that for optimal
binding the PENAM should have a conformation in which the
nucleotidic bases are stacked approximately at the 3.5
Angstrom interval and are oriented away from the backbone
5   in such a manner that the hydrogen bond donors and
acceptors of the nucleotidic bases can directly "read"
the complementary bases on the target nucleic acids by
specific hydrogen bonding interactions.  The differences
can be visualized most readily when the PENAMs are viewed
10  in beta-sheet-like extended conformation.  <u>Figure 2a</u>
presents a "side view" illustration of the two versions.
The differences in estimated orientation are even more
striking when viewed from the "top" (i.e. along the
length of the polymer) as illustrated in <u>Figure 2b</u>.  In
15  particular, in Version I, the adenine moieties in the
side chains were approximately 180° apart on the alpha
carbons of each pair of adjacent monomers and, as a
result, the adenine moieties could not be effectively
stacked or aligned at the interval of 3.5 Angstrom
20  spatial distance.  In contrast, in PENAM Version II, the
adenine moieties were much closer to each other in space
(at an angle of approximately 65°) and could therefore be
stacked at a spatial distance of approximately 3.7
Angstrom, which substantially enhances their ability to
25  hybridize with a target nucleic acid.

Similar molecular models were constructed using
other nucleic bases (e.g., B = thymine, guanine,
cytosine, uracil), and confirmed the preceding results.


30                        <u>Example 2</u>
<u>Use of molecular modelling to predict homomorphism</u>
<u>between PENAMs of the present invention and target DNA</u>

As an illustration of the use of molecular modelling
to assess the likely homomorphism between a particular
35  version of the PENAMs and a target nucleic acid, we used
the molecular modelling techniques referred to in Example
1 to assess several types of PENAMs having varying spacer

49

groups.  In particular, we examined models of a single
strand of DNA (a thymine 12-mer) in association with
complementary PENAMs having NuAA monomers of the
following formula:

5

$$
\begin{array}{ccc}
 & & B \\
 & & | \\
 & W & S3 \\
 & | & | \\
--N--S1--E--S2--C-- \\
 & | & \| \\
 & Y & X
\end{array}
$$

10
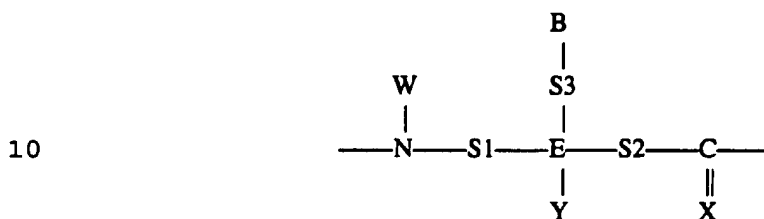
15     in which E is carbon; W is hydrogen; Y is hydrogen;
S1 is a bond; S3 is an ethylene group; X is oxygen; B is
adenine; N is nitrogen; and wherein S2 was selected from
the group consisting of a bond, a methylene group, an
ethylene group and a propylene group.

20     Energy minimization (100 cycles) was performed using
Tripos force field parameters provided in the Sybyl
software package.  Following the teachings of the present
invention, the energy minimization was carried out in a
manner that allowed for the introduction of varying
25  chiral centers in the peptidic backbone of the PENAM such
that the overall energy state of the molecule was
minimized.

The resulting energy-minimized models are shown in
Figure 3(a-d).  Although all of the models exhibited the
30  formation of double helices between the PENAM and the
target DNA, the modelling predicted an increasing
homomorphism (which appears as an increasingly regular
helicity) when the S2 spacer group was increased in
length from a bond (Figure 3a), to a methylene group
35  (Figure 3b), to an ethylene group (Figure 3c), and
finally to a propylene group (Figure 3d).  The
corresponding model for an interaction between two
complementary strands of DNA is shown in Figure 3e.
While the increase from methylene to ethylene to

propylene is believed to be advantageous, further increasing the length of the S2 group (beyond that of a three-atom bridge as in propylene) is believed to be less advantageous.

Additional estimates of the relative homomorphism were obtained by assessing the total energy for the resulting molecules. The total energy was estimated as a sum of bond stretching energy, angle bending energy, torsional energy, out of plane bending energy and van der Waals energy, using the same molecular modelling software. Consistent with the apparently enhanced helicity, as described above, the resulting total energy estimates revealed a considerable decrease in total energy (suggesting an increase in homomorphism) when the length of the S2 spacer group was increased from 0 (in the case of a bond), to 1 (methylene), to 2 (ethylene), to 3 (propylene).

In all cases, the energy-minimized structures incorporated D-chiral centers into the peptidic backbone. For example, for the particular PENAMs modeled in this illustration, the following D-containing sequences were obtained after 100 cycles of energy minimization: DLLLDDDDDLL in Figure 3a; LDDDDDDDDDDD in Figure 3b; DDDDDDDDDDDD in Figure 3c; and DDDDDDDDDDDD in Figure 3d. These modeling data, as well as physical data involving PENAMs with alternating D- and L-chiral centers described below, suggest that the ability to incorporate D-chiral centers into the PENAM can be used to substantially enhance the homomorphism between particular PENAMs and their target nucleic acids.

51

## Example 3

### Use of retrosynthesis and molecular modelling to identify synthetic schemes for the preparation of NuAA monomers

The NuAA monomers of the present invention can be
5    synthesized from available starting compounds, such as
amino acids, via a number of different synthetic
pathways. As described herein, the preferred NuAA
monomers contain a nucleic base that is substituted on
the side chain at a distance which is similar to that
10   between the nucleic base and the sugar-phosphate backbone
in standard ODNs. Molecular modelling and retrosynthesis
techniques have been used to identify
conveniently-prepared intermediates and corresponding
synthetic steps for the production of NuAA monomers of
15   the present invention. An example of such a
retrosynthetic analysis has been illustrated in Figure
4a, as is described in more detail below.

We have used these techniques to develop a
convergent modular synthetic approach for the synthesis
20   of these stereo-specific PENAMs via NuAA monomers. This
strategy includes a basic set of chemical building blocks
of the structural units which allow for the synthesis of
a diverse class of novel chemotherapeutic agents in an
efficient and cost effective manner. The chemistry
25   involved in the synthesis of these key intermediates is
fairly easy, extensively studied and is well established
in the literature [63-65, 278, 279]. As described
herein, the preferred building blocks include: (i) NuAA
monomers and their derivatives; (ii) amino acids (AA) and
30   their derivatives; and (iii) aza-amino acids and their
derivatives.

52

## Example 4

### Use of α-amino-α-carboxylic acids as substrates for the preparation of NuAA Monomers

As defined earlier, NuAA monomers are amino acids
that have purine/pyrimidine nucleic bases or their
heterocyclic analogs substituted in the aminoacyl side
chain depending from the alpha carbon.  These key
intermediates can have L- or D-configurations at the
alpha carbon (except for the aza-NuAA monomers, in which
case the α-carbon is replaced by nitrogen, as described
herein).  The distance between the nucleic base and the
peptidic backbone can be easily manipulated by varying
the length of the side chain of these nucleicamino acids,
i.e., the S3 position in the formula of the NuAA monomer.
Molecular modelling has been used to show that, in order
to mimic the homomorphism of native nucleic acids, the
nucleic base is preferably sustituted on the side chain
at least two carbon units distance from the peptidic
backbone (e.g., γ-substituted), and is preferably not
more than four carbon units away from the backbone.
Thus, S3 is preferably a spacer group with a backbone of
at least 2 atoms, preferably at least three atoms.  Such
NuAA monomers can be easily synthesized from commercially
available starting materials, for example, natural
alpha-amino acids (an illustrative example of the
retrosynthesis of PENAMs and NuAA monomers from α-amino-
α-carboxylic acids is shown in **Figure 4a**).  These natural
amino acids provide a rich source of diverse chemical
functionality, which are convenient for suitable chemical
transformations using well-known synthetic techniques;
and, in addition, they also serve as chiral templates by
virtue of the chiral center generally present at the
alpha-carbon.  As discussed herein, the use of chiral
monomers to introduce varying centers of chirality (or
"antipodes") into the peptidic backbone allows for the
production of PENAMs that are homomorphic to their target

nucleic acids, allowing for efficient stacking and hydrogen bonding.

The purine/pyrimidine nucleic bases or their heterocyclic analogs can be introduced as aliphatic, acyl, amide or amine side chain substituents by chemical manipulations of these alpha-amino acids (as described herein). Especially convenient precursors for such manipulations are "omega"-halogenated (i.e. terminally halogenated) amino acids. Thus, as illustrated in Figure 4a, NuAA monomers can be readily synthesized from ω-halogenated amino acids and any nucleic base. These ω-substituted haloamino acids can themselves be easily synthesized from commercially available natural amino acids, as described below.

## Example 5

### Preparation of haloamino acids (XAAs)

Based on the retrosynthetic analysis illustrated above, haloamino acids (XAAs) have been identified as convenient chemical building blocks in the synthesis of NuAA monomers. These haloamino acids are modified amino acids in which at least one of the hydrogens on the side chain carbons has been substituted by a halogen group (especially Cl, Br, I). The side chain distance can be readily varied by substituting the halogen group at, e.g., the $\beta$-, $\gamma$-, $\delta$- or $\omega$- carbon in the side chain. Subsequent nucleophilic substitution of the halogen group by a nucleic base gives ready access to a wide variety of NuAA monomers, which can then be used to prepare stereo specific $\alpha$-amide linked PENAMs using synthetic techniques analogous to those described in, e.g., references [21-25]. The molecular modelling analyses discussed above suggested that NuAA monomers in which the nucleic base is either at $\gamma$ (C3) or $\delta$ (C4) carbon atoms in the side chain are likely to position and orient the nucleic base at particularly effective distances from the peptidic backbone.

54

Although any halo derivative, including chloro-, bromo- or iodo-amino acids is suitable for synthesis of the NuAA monomers, bromo-derivatives were selected as an initial example because there are a number of well-known synthetic routes available for the preparation of brominated amino acids [278, 279]. Of those routes, the conversion of ω-hydroxyamino acids to corresponding ω-bromoamino acids appeared to be especially convenient. In particular, several brominating reagents such as hydrobromic acid, phosphorous tribromide, phosphorous pentabromide, thionyl bromide, methanesulfonybromide and carbon tetrabromide/tertiary organophosphine have been used effectively for the bromination of hydroxy compounds (primary and secondary hydroxyl groups) to bromides [278, 279]. The use of carbon tetrahalide-organophosphine is advantageous for converting primary hydroxyl groups to corresponding halides [280]. An illustrative retrosynthetic scheme is shown in Figure 4b.

As will be appreciated by those of skill in the art, advantageous reagents are characterized by simplicity of experimental procedure, good yields, and relatively mild, essentially neutral reaction conditions. In addition, a number of these hydroxyamino acids are commercially available or can be easily prepared in large quantities from their corresponding dicarboxylic esters by reduction with common reducing agents [278, 284]. Accordingly, γ-brominated-α-aminobutyric acid (4-bromo-2-amino-butanoic acid) and its homologue, δ-brominated-α-amino-valeric acid (5-halo-2-amino-pentanoic acid) were identified as particularly convenient intermediates. By way of illustration, the preparation of 4-bromo-2-amino-butanoic acid is described below.

In addition to the halogenation of hydroxy compounds, halogenation of ω-lactones is another useful synthetic methodology for the preparation of halogenated

carboxylic acids and their derivatives [22-25, 279, 281, 282].


## Example 6

### Synthesis of 4-bromo-2-amino-butanoic acid

For use in the synthesis of NuAA monomers, we prepared enantiomerically pure 4-bromo-2-amino-butanoic acid as L-, D-, and racemic D/L- stereoisomers, suitably protected at their amino and carboxylic acid functional groups in order to facilitate their use in existing solution and solid phase peptide synthesis procedures. As described earlier, 4-bromo-2-amino-butanoic acid can be conveniently prepared from an optically active 4-hydroxy-2-amino-butanoic acid (homoserine) by carbon tetrabromide-triphenylphosphine. However, if the starting material homoserine is not appropriately protected at carboxyl and amino groups, then the chemical conversion can lead to a cyclized product ($\alpha$-amino-$\gamma$-butyrolactone) instead of 4-bromo-2-amino-butanoic acid. In addition, protection of the $\alpha$-amino and $\alpha$-carboxyl groups of homoserine can be affected by its tendency to lactonize. This problem can be readily circumvented by using $\alpha$-amino-$\gamma$-butyrolactone as the starting material. The racemic and L-isomer of 4-bromo-2-amino-butanoic acid have been prepared as hydrogen bromide salts in good yield and high optical purity from their corresponding $\alpha$-amino-$\gamma$-butyrolactones by bromination with hydrogen bromide in acetic acid [281]. Although $\alpha$-amino-$\gamma$-butyrolactone is readily available only as a racemate, $\alpha$-amino-$\gamma$-butyrolactone can be synthesized in all stereoisomeric forms either from the corresponding homoserine ($\gamma$-hydroxy-$\alpha$-amino-butanoic acids) or from the corresponding methionine [283]. Even though both D- and L-homoserine are commercially available, their costs currently make them less desirable as starting materials. Alternatively, D- and L-homoserine can be easily synthesized from readily

56

available D- and L- aspartic acids, respectively.
Reduction of the β-carboxyl group of appropriately
protected D- and L- aspartic acid can provide
corresponding protected homoserine derivatives in gram
5  quantities [284], which in turn can be cyclized in the
presence of mineral acid (hydrochloric acid) to yield the
required lactones.

We used methionine instead of aspartic acid as the
starting material for the synthesis of
10  α-amino-γ-butyrolactone since the D/L-, D- and L-
stereoisomers of methionine are readily available as
inexpensive chiral amino acids and can be converted in
one step to the corresponding D/L-, D-, and
L-α-amino-γ-butyrolactones in high enantiomeric purity
15  and in excellent yields [283].

All the three stereo isomers, D/L-, D-, and
L-α-amino-γ-butyrolactones were synthesized in about 85%
yield, by modifying the literature procedure of Natelson
[283], as described below.

20

### Example 7
### Synthesis of the NuAA monomer 4-(N9-adeninyl)-
### 2-(t-butyloxycarbonylamino)-butanoic acid

As an illustration of the synthetic procedures, we
25  synthesized D/L-, L- and D- stereo isomers of the NuAA
monomer, 4-(N9-Adeninyl)-2-(t-butyloxycarbonylamino)-
butyric acid [(D/L)-AA(Boc)GAdBA = AdhAla], in good yield
and high optical purity (i.e., greater than 99%), using
D/L-, L- and D- methionine as the chiral precursors,
30  respectively. The synthetic procedure is illustrated in
Figure 5, showing the synthesis of racemic NuAA monomers
[(D/L)-AA(Boc)GAdBA = AdhAla] using D/L-methionine as the
starting material. The structures of the various
intermediates were confirmed using standard techniques
35  including ^{1}H NMR, ^{13}C NMR, specific rotation and mass
spectrometry.

57

To summarize this illustrative synthetic procedure,
D/L-methionine, on alkylation at the methylmercapto group
with bromomethylacetic acid or its ester, formed
corresponding sulfonium salts in situ, which on heating

5  underwent nucleophilic attack by water to eliminate
methanethiomethylacetate to give corresponding homoserine
in situ.  This homoserine on further heating at high
temperature (>90°C) cyclized to yield the known
intermediate α-amino-γ-butyrolactone hydrobromide salt

10  (AAGBL•HBr) in about 85-90% yield.  By following the
literature procedure [282], this lactone, on treatment
with 35% HBr-HOAc at about 75 degrees Celsius, gave
corresponding γ-bromoamino acid hydrobromide salt
(AAGBBA•HBr).  The α-amino group and the α-carboxylic

15  group need to be protected to prevent them from reacting
with the γ-bromo group and causing any side reactions
during the subsequent synthesis.  The α-carboxylic group
of γ-bromoamino acid•HBr was protected as its methyl
ester (AAGBMB•HCl) by passing dry HCl gas into a

20  methanolic solution, while the α-amino group was
protected as a carbamate by treating the methyl ester•HCl
with t-butyloxy carbonate in the presence of aqueous
$NaHCO_3$ to yield 4-bromo-2-(t-butyloxycarbonylamino)-
methylbutyrate, [AA(Boc)GBMB].  The NuAA monomer

25  (D/L)-4-($N^9$-adeninyl)-2-amino($^t$Boc)-butanoic   acid
[(D/L)-AA(Boc)GAdBA = AdhAla] was prepared essentially in
accordance with these known synthetic procedures [21].
The reaction of this bromo carbamate [AA(Boc)GBMB] with
adenine  in the presence of dry potassium carbonate and

30  DMF as a solvent gave approximately 70% yield of fully
protected NuAA monomer [(D/L)-AA(Boc)GAdMB].

As shown in Figure 6, NuAA monomers in which the
nucleic base is other than adenine (A) can be prepared
similarly   by   reacting   4-bromo-2-(t-butyloxy-

35  arbonylamino)-methylbutyrate [AA(Boc)GBMB]  with  the
appropriatly protected nucleic base, such as guanine (G),

58

cytosine (C), thymine (T), uracil (U) or their heterocyclic analogs.

The individual chemical reactions sumarized above were carried out essentially as described in the
5   literature. The following detailed description of the individual steps is provided for the further convenience of the reader.

(1) **Synthesis of (D/L)-α-Amino-γ- butyrolactone**
10  **hydrobromide [(D/L)-AAGBL●HBr]**:

(D/L)-α-Amino-γ-butyrolactone hydrobromide [(D/L)-AAGBL●HBr] was synthesized from (D/L)-methionine by following the modified literature procedure referred to above. To an aqueous ethanolic solution (1:1, 200
15  mL), (D/L)-methionine (0.1M, 14.92g) and methylbromoacetate (0.11M, 17.17g) were added at room temperature. The resulting suspension was heated to 55-60°C in an oil bath until it became a homogeneous solution (about 1.5-2 hrs). It was left at that
20  temperature overnight (12 hrs). After 12 hrs the resulting dark yellow solution was heated at reflux for about 3-5 hrs. The thin layer chromatograph (tlc) (n-BuOH:H₂O:HOAc=4:1:1) of this reaction mixture showed that the reaction had gone to completion. The dark brown
25  solution was cooled to room temperature and evaporated to dryness under vacuum using a water aspirator. To this viscous dark brown residue a mixture of isopropanol and toluene (1:1, 50 mL) was added, and the last traces of water were removed by evaporating the resulting solution
30  to dryness. The precipitated, off white crystalline solid was suspended in cold absolute ethanol (50 mL), filtered off, washed thoroughly with a mixture of cold ethanol and toluene (1:1, 50 mL) and air dried. The filtrate and the washings were combined and re-evaporated
35  to generate a viscous liquid which was stirred with 4M HCl in dioxane (60 mL) at -40°C for 4-6 hrs. It was left in a refrigerator overnight. The second crop of the

59

precipitated white crystals were filtered off, washed
thoroughly with cold ethanol and air dried.  The overall
yield of the product (D/L)-AAGBL●HBr was about 15-15.5 g.
(80-85%).  Analogously, the enantiomers (L)-AAGBL●HBr and

5    (D)-AAGBL●HBr were synthesized from (L)-methionine and
(D)-methionine,  respectively,  and  were  purified  by
recrystallization from 10% aqueous ethanol in about 75%
yield.  The structures of all of the stereoisomers were
confirmed by $^1$H, $^{13}$C NMR, specific rotation and mass

10   spectrometry.


(2)   Synthesis  of  (D/L)-α-Amino-γ-bromo  butyric  acid
Hydrogen bromide [(D/L)-AAGBBA●HBr]:

(D/L)-AAGBL●HBr  (0.05M,  9.101g,  Aldrich  Chemical

15   Co.) was heated with freshly made 35% HBr-HOAc solution
(10 equiv, 0.5M) in a teflon vessel containing a steel
bomb at 75°C for 3 hrs.  The steel bomb was cooled to
room temperature.  The reaction mixture had precipitated
and formed white crystals.  This slurry was transferred

20   to a flask and the contents were evaporated at 40°C using
a water aspirator vacuum to remove excess HBr and HOAc.
The  resultant  yellowish  white  crystalline  solid  was
suspended  in  a  1:1  mixture  of  MeOH  and  toluene  and
evaporated again to remove traces of HOAc.  The yellowish

25   white crystalline solid was resuspended in diethylether,
filtered off, washed thoroughly with diethyl ether and
air dried to give white crystalline product in almost
quantitative yield (>95%).  The proton NMR of this crude
product showed that about 10-15% of the lactone remained

30   unreacted.  The product bromoamino acid, (D/L)-AAGBBA●HBr
could be recrystallized from absolute ethanol but it was
used without any further purification for the next step.
The  enantiomers  (L)-AAGBBA●HBr  and  (D)-AAGBBA●HBr
were  synthesized  from  (L)-AAGBL●HBr  and  (D)-AAGL●HBr,

35   respectively, by following the same procedure and were
recrystallized from absolute ethanol.  The structures of

60

all of the stereoisomers were confirmed by $^1$H, $^{13}$C NMR, specific rotation and mass spectrometry.

(3)   Synthesis   of   (D/L)-α-amino-γ-bromomethylbutyrate
5 hydrogen chloride [(D/L)-AAGBMB●HCl]:

The methyl ester (D/L)-AAGBMB●HCl was prepared in quantitative yield by dissolving the bromoamino acid, (D/L)-AAGBBA●HBr (0.05M, 13.15g) in an excess of absolute methanol (5mL/mM) and bubbling dry HCl gas through this
10 methanolic solution for about 2 hrs while maintaining the reaction temperature between 35-40°C. The light yellow solution was evaporated below 40°C using a water aspirator vacuum to remove th excess HCl gas and methanol. The residual yellow liquid was diluted with a
15 mixture of methanol and toluene (1:1, 50mL) and evaporated again to dryness to give a syrupy liquid which on further drying at high vacuum gave ivory-white crystals of bromoamino methyl ester, (D/L)-AAGBMB●HCl, which could be purified by crystallization from
20 CHCl$_3$-diethyl ether mixture. For practical purposes, the product was pure enough for the ensuing step.

The enantiomers (L)-AAGBMB●HBr and (D)-AAGBMB●HBr were synthesized similarly from (L)-AAGBBA●HBr and (D)-AAGBBA●HBr respectively and were recrystallized from
25 absolute ethanol and diethylether as co-solvent. The structures of all of the stereoisomers were confirmed by $^1$H, $^{13}$C NMR, specific rotation and mass spectrometry.

(4)   Synthesis    of    (D/L)-α-(t-Boc)amino-γ-bromo
30 methylbutyrate [(D/L)-AA(Boc)GBMB]:

The carbamate, (D/L)-AA(Boc)GBMB, was prepared by treating the haloamino ester, (D/L)-AAGBMB●HCl with t-butyloxycarbonate in the presence of NaHCO$_3$. To an aqueous solution of (D/L)-AAGBMB●HCl (0.05M, 11.63g; 15mL
35 H$_2$O), an aqueous solution of NaHCO$_3$ (0.11M, 9.24g, 110 mL H$_2$O) was added at 0°C. After the initial evolution of CO$_2$ gas, a solution of t-butyloxycarbonate (0.055M, 12g) in

61

p-dioxane (65 mL) was added.   The resulting reaction mixture was vigorously stirred at 0°C for an hour and then warmed up to room temperature gradually.   The reaction mixture was stirred at room temperature and was

5   monitored by thin layer chromatography.   The reaction went to completion within 4-6 hrs.  The precipitated oily liquid (denser than water) was separated and the supernatant was extracted with chloroform.   The chloroform extract was combined with the oil liquid and

10  was then thoroughly washed with water.   The organic extract after evaporation at room temperature provided a light yellow oil with characteristic odor of tBuOH.  The product (D/L)-AA(Boc)GBMB was purified as white crystals in about 85% yield by flash chromatography using a

15  gradient of 7.5-15% ethylacetate (EA) in hexane.   It could also be crystallized from diethyl ether-petroleum ether mixture.

The enantiomeric methyl esters, (L)-AA(Boc)GBMB and (D)-AA(Boc)GBMB, were synthesized similarly from

20  (L)-AAGBMB●HBr and (D)-AAGBMB●HBr, respectively, and were recrystallized from a diethyl ether and petroleum ether as co-solvents.   The structures of all of the stereoisomers were confirmed by $^1$H, $^{13}$C NMR, specific rotation and mass spectrometry.

25

(5)   <u>Synthesis   of   (D/L)-α-Amino(tBoc)-γ-(N9)-adeninyl methylbutyrate  [(D/L)-AA(Boc)GAdMB]</u>:

To an oven-dried flask containing a suspension of adenine (0.025M, 3.378g) in freshly distilled

30  dimethylformamide (DMF) (90mL; 3.5 mL/mM base), anhydrous K$_2$CO$_3$ was added.  This mixture was stirred for 15 min at room temperature and (D/L)-AA(Boc)GBMB (0.025M, 7.4g) was added in 25 mL dry DMF under an inert atmosphere.   The reaction was monitored by thin layer chromatography and

35  the condensation went to completion by leaving the reaction mixture stirred under inert atm at room temperature over 72 hrs.   The reaction mixture was

62

filtered off to remove the precipitated salts (KBr,
KHCO$_3$). The filter cake was thoroughly washed with DMF.
The washings were combined with the filtrate and the
resulting solution was evaporated to dryness to give a
5    slurry containing precipitated solid. This viscous
liquid was diluted with CHCl$_3$ (50 mL) and was filtered to
remove the solid. The organic filtrate was thoroughly
washed with water, dried over Na$_2$SO$_4$ and evaporated to
give a yellowish liquid. The N9-substituted product
10   (D/L)-AA(Boc)GAdMB, was purified by flash chromatography
using 4-7% MeOH-EA solvent system in about 80-85% yield.


      The adeninyl carbamate methylester enantiomers,
(L)-AA(Boc)GAdMB and (D)-AA(Boc)GAdMB were synthesized
15   similarly from the corresponding bromocarbamate esters,
(L)-AA(Boc)GBMB   and   (D)-AA(Boc)GBMB   and   adenine,
respectively. The structures of all of the stereoisomers
were confirmed by $^1$H, $^{13}$C NMR, specific rotation and mass
spectrometry.
20

      (6)   Synthesis   of   (D/L)-α-Amino(tBoc)-γ-(N9)-adeninyl
butyric acid [(D/L)-AA(Boc)GAdBA]:
      The hydrolysis of the methyl ester was carried out
to provide the corresponding tBoc-protected amino acid as
25   a building block for the solid phase peptide synthesis.
The methyl ester (D/L)-AA(Boc)GAdMB (0.01M, 3.50g) was
dissolved in aqueous dioxane (1:1), and the clear
solution was chilled to 0° to 5°C. To this cold solution
an aqueous solution of LiOH•H$_2$O (0.0115 M, 0.5 g) was
30   added. The hydrolysis was complete within 3 hrs. of
stirring the solution below 5°C. The reaction solution
was evaporated on a rotovap below 5°C and the last traces
of water and dioxane were removed azeotropically using
benzene as co-solvent. The white solid that was obtained
35   was redissolved in a minimum amount of aqueous dioxane
and then acidified to pH 4-6 with HCl-dioxane solution.
The precipitated white solid was filtered, washed with

63

dioxane and dried under high vacuum.  The yield of the nucleic aminoacyl monomer (D/L)-AA(Boc)GAdBA was about 90-95%.

5     The nucleic aminoacyl enantiomers (L)-AA(Boc)GAdBA and (D)-AA(Boc)GAdBA were prepared similarly by hydrolysis of the corresponding methylesters. (L)-AA(Boc)GAdMB and (D)-AA(Boc)GAdMB. The structures of all of the stereoisomers were confirmed by $^1$H, $^{13}$C NMR, specific rotation and mass spectrometry.

10

Example 8

Synthesis of NuAA monomers derived from α-amino-(β-/γ-/δ-),α-dicarboxylic acids:

      α-amino-(β-/γ-/δ-), α-dicarboxylic acids offer the
15    unique advantage of forming amide bonds (peptide bonds) either at α-carboxylic group or at β-/γ-/δ- carboxylic group.    As    a    consequence    α-amino-(β-/γ-/δ-), α-dicarboxylic acids can be used to introduce flexibility via β-/γ-/δ- amide linkages in the peptide backbone over
20    α-amino-α-carboxylic acids. The α-carboxylic acid group could be chemically manipulated to a suitable side chain, on which the nucleic base is introduced at a homomorphic distance. Various synthetic transformations known in the art can be utilized to prepare these NuAA monomers
25    [63-65]. As an example, a retrosynthetic strategy has been illustrated in Figure 7.

Example 9

Synthesis of aza-NuAA monomers:

30    Aza-NuAA monomers can be derived from nucleic "aza-amino acids". The nucleic aza-amino acids can be easily prepared from aza-amino acid derivatives, such as semicarbazides (aza-amino acid amides) and carbazic acid (hydrazinecarboxylic acid) esters. Usually the aza-amino
35    acid is relatively unstable unless protected at the carboxylic group, due to the fact that N-carboxylic acids are readily decarboxylated. Various synthetic methods,

64

known in the art can be utilized to synthesize these aza-amino acid derivatives [68]. Preparation of aza-amino acid derivatives can be based on hydrazine chemistry. In general, a nucleic base or its analog with suitable
5  linker is condensed with hydrazine or a protected hydrazine. This alkylated or nucleic base substituted hydrazine is then treated with an appropriate "carbonyl donor" such as carbonochloridic esters or carbonic diesters or haloesters. Such a retrosynthetic strategy
10  is illustrated in Figure 8.


## Example 10
### Assembly of PENAMs from NuAA monomers via solid phase peptide synthesis

15       Protected NuAA monomers, as described in Example 7, can be oligomerized using the t-butyloxycarbamate (tBoc) strategy on the automated peptide synthesizer to prepare PENAMs of the desired chain length. To summarize, this strategy requires deprotection of the methyl ester to
20  free the carboxylic acid. The methyl ester (D/L)-AA(Boc)GAdMB was hydrolyzed by LiOH to yield t-BOC protected NuAA monomer, (D/L)-AA(Boc)GAdBA. Similarly, (L)-4-(N9-adeninyl)-2-amino(tBoc)-butyric acid, [(L)-AA(Boc)GAdBA] and (D)-4-(N9-adeninyl)-2-amino(tBoc)-
25  butyric acid [(D)-AA(Boc)GAdBA] have also been synthesized from (L)-methionine and (D)-methionine, respectively.
        The following is an even more detailed description of the individual synthetic steps:
30       All three stereoisomers, D/L-, L- and D- of the unnatural NuAA monomer 4-(N9-Adeninyl)-2-amino-butyric acid [also abbriviated as GAdAABA or AdhAla] and their derivatives were synthesized in the laboratory in enantiomerically pure form, as described in the preceding
35  examples. All other amino acid derivatives were of the L-configuration unless stated, purchased either from Applied Biosystems Inc. (ABI) or Bachem Inc. and were

65

checked for purity by thin layer chromatography, melting
point, and optical rotation.  p-methylbenzhydrylamine
(MBHA)    resin,    (copolystyrene-1%    divinylbenzene,
0.69meq/g, 100-200 mesh) was purchased from Peninsula

5    Laboratory, CA.  All solvents were purchased either from
ABI or Burdick and Jackson (distilled in glass grade).
Dimethylformamide (DMF) and disopropylethylamine (DIPEA)
were distilled from ninhydrin, followed by calcium
hydride at reduced pressure and stored over molecular

10   sieves (Linde, 4 Angstrom).  Boc deprotection reagent,
trifluoroacetic   acid   (TFA),   and   peptide   coupling
reagents,      dicyclohexylcarbodiimide      (DCC),
1-hydroxybenzotriazole (HOBT) were purchased from ABI and
were used as supplied.  1-Benzotriazolyloxy-

15   tris(dimethylamino)-phosphonium hexafluorophosphate (BOP)
and    O-(benzotriazol-1-yl-)-1,1,3,3-tetramethyluronium
hexafluorophosphate (HBTU) were purchased from Richielu
Chemicals  Co.,  Canada.   1-Hydroxy-7-azabenzotriazole
(HOAT) was either synthesized in the laboratory or was

20   purchased from the Miligen Corporation.

Solid phase peptide synthesis was carried out on an
Applied   Biosystem   Inc.   (ABI)   automated   peptide
synthesizer, model 431A, using standard BOC chemistry
protocols.   In order to obtain the end product as an

25   amide   at   the   C-terminus   after   HF   cleavage,
p-methylbenzhydrylamine (MBHA) resin was used as a
polymeric support to anchor the growing peptide chain.
The peptide synthesizer was operated in a semi-automated
manner and all the cycles were modified according to the

30   scale of the reaction and to facilitate the completion of
each coupling reaction.

The PENAMs were synthesized with L-lysine residues
flanking both the N-terminus and the C-terminus because
they are believed to enhance the solubility of the

35   resulting PENAM in aqueous solutions.

A racemic PENAM [H-Lys-{(D/L)-(AhAla)$_{10}$}-Lys-NH$_2$] was
synthesized from 4-(N9-adeninyl)-2-amino-butyric acid

66

[(D/L)-GAdAABA] and L-lysine on a peptide synthesizer on
0.2 mM scale. DCC and HOBT were used as the coupling
reagents to couple the lysine residues whereas BOP and
HOBT were used to couple the nucleic aminoacid monomers.

5    As anticipated, the purification of this racemic
PENAM resulted in the generation of a large number of
diastereomers ($2^{10}$ = 1024), which were isolated as a
mixture by HPLC on C-18 reverse phase column. The
molecular weight of this PENAM was in accordance with its
10   calculated mass as determined by mass spectrometry.

Similarly, the enantiomerically pure PENAMs
[H-Lys-{-(D-AdhAla)-(L-AdhAla)-}$_5$-Lys-NH2]     and
[H-Lys-(L-AdhAla)$_{10}$-Lys-NH2] were synthesized from the
corresponding nucleicamino acids, (D)-4-(N9-adeninyl)-2-
15   amino-butyric acid [(D)-GAdAABA or (D)-γ-adeninyl-α-
amino-butyric acid] and (L)-4-(N9-adeninyl)-2-amino-
butyric acid [(L)-GAdAABA or (L)-γ-adeninyl-α-amino-
butyric acid] and (L)-lysine on 0.2 mM scale, by
following the protocols used for the racemic PENAM,
20   [H-Lys-{(D/L)-(AhAla)$_{10}$}-Lys-NH$_2$]. The only difference was
the use of a new coupling reagent HOAT in combination
with HBTU which is believed to increase the coupling
yield and to minimize racemization at the alpha-carbon
[285].

25

## Example 11
### Binding of a PENAM to a target nucleic acid

Thermal denaturation of an ordered, native structure
of a nucleic acid disrupts the base-pair stacking and, as
30   a result, the UV absorbance increases. The change in UV
absorbance, known as hypochromicity, is a measure of
base-pairing and base-stacking between the two
complementary strands. The UV absorbance profile as a
function of temperature is called a melting curve and the
35   midpoint of this curve is defined as the melting
temperature, $T_m$, at which 50% of the double strand is
dissociated into its two single strands. Thus, the

measurement of UV absorbance melting curves provides qualitative and quantitative structural information about the nucleic acid bound to its complementary ligand. $T_m$ also depends on the concentration of the oligonucleotide
5  and the properties of the solvent (buffer: pH, ionic strength, etc.).

Binding studies were carried out by hybridizing the isolated PENAM described above to its complementary oligonucleotide "$dT_{10}$", followed by thermal denaturation
10  and measurement of UV absorbance as a function of temperature. The synthetic oligodeoxynucleotide (ODN) $dT_{10}$ and its complementary oligodeoxynucleotide $dA_{10}$ were used as the reference nucleic acids. The samples prepared in phosphate buffer ($Na_xPO_4$) can precipitate
15  magnasium phosphate at high temperature during the thermal denaturation cycle. This problem was readily avoided by repeating the experiment in 10mM sodium cacodylate buffer ($Me_2AsO_2Na$) instead of the phosphate buffer. The results are summarized in **TABLE 1**.

20

**TABLE 1:**

| Reference Strand | Complementary Strand | | Stoichio-metry | | | | | $T_m$ (°C) | |
|---|---|---|---|---|---|---|---|---|---|
| dNu | dNu | pNu | | | | | | $Na_xPO_4$ | $Me_2AsO_2Na$ |
| $dT_{10}$ | $dA_{10}$ | - | 1 | : | 1 | : | 0 | 28.045 | 29.41 |
| $dT_{10}$ | - | $KpA_{10}K_m$ | 1 | : | 0 | : | 1 | 31.26 | 31.00 |
| $dT_{10}$ | $dA_{10}$ | $KpA_{10}K_m$ | 1 | : | 1 | : | 1 | 31.41 | 31.052 |
| $dT_{10}$. | - | $2KpA_{10}K_m$ | 1 | : | 0 | : | 2 | 30.8 | 32.57 |
| $dT_{10}$ | $dA_{10}$ | $2KpA_{10}K_m$ | 1 | : | 1 | : | 2 | 30.07 | 32.26 |
| $2dT_{10}$ | - | $KpA_{10}K_m$ | 2 | : | 0 | : | 1 | - | 32.28 |
| - | - | $2KpA_{10}K_m$ | 0 | : | 0 | : | 1 | no real transition | no real transition |

35
dNu = deoxyoligonucleotide; pNu = nucleic oligopeptide;
$pA_{10}$ = PENAM A10; $K_m$ = lysine amide at the C-terminus.

68

These data confirm that the PENAM binds to its complementary nucleic acid strand, and that it exhibits a real transition from an ordered structure to a
5   disordered one on thermal denaturation.

Strikingly, the PENAM was observed to melt at an even higher temperature than the reference ODN, $dT_{10}:dA_{10}$. Although the increase in melting temperature was somewhat modest, it is a significant result because it shows that,
10  in spite of being a complex diastereomeric mixture, the PENAMs recognized and bound quite effectively to their complementary oligodeoxynucleotide strands. It is thus expected that certain stereospecific PENAMs will bind to their complementary oligonucleotide targets even more
15  tightly than the observed average for the mixture.

In addition, the binding affinity of these PENAMs can be further enhanced by introducing modified nucleic bases which can improve base pairing by forming additional hydrogen bonds or by conjugation with known
20  nucleic acid intercalating agents such as acridines, anthraquinones, phenanthridines, phenazines, as described above.

69

**References**:

1.    Miller, P. S. and Ts'o, P. O. P. "Oligonucleotide Inhibitors of Gene Expression in Living Cells: New
5   Opportunities in Drug Design". *Ann. Rep. Med. Chem.* **23**: 295-304, **1988**. Review.

2.    Matteucci, M. D. and Bischofberger, N. "Sequence-defined Oligonucleotides as Potential
10  Therapeutics". *Ann. Rep. Med. Chem.* **26**: 287-296, **1991**. Review.

3.    Cohen, J. S. Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. Topics in Molecular and
15  Structural Biology. 1-255, **1989**, CRC Press, Boca Raton, Florida.

4.    Ghosh, M. K. and Cohen, J. S. "Oligodeoxynucleotides as antisense inhibitors of gene expression". *Prog. in Nu.*
20  *Acid Res. and Mol. Biol.* **42**(79): 79-126, **1992**. Review.

5.    Helene, C. and Toulme, J.-J. "Specific Regulation of Gene Expression by Antisense, Sense and Antigene Nucleic Acids". *Biochim. Biophys. Acta.* **1049**: 99-125, **1990**.
25  Review.

6.    Stein, C. A. and Cohen, J. S. "Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review". *Cancer Res.* **48**: 2659, **1988**. Review.
30
7.    Stein, C. A. and Cheng, Y. "Antisense Oligonucleotides as Therapeutic Agents-Is the Bullet Really Magic?". *Science.* **261**: 1004-1011, **1993**. Review.

35  8.    Uhlmann, E. and Peyman, A. "Antisense Oligonucleotides: A New Therapeutic Principle". *Chem. Rev.* **90**(4): 544-579, **1990**. Review.

70

9.   Goodchild, J. "Conjugates of oligonucleotides and modified oligonucleotides: a review of their synthesis and properties". *Bioconjug Chem.* **1**(3): 165-87, **1990**. Review.

10.  Stein, C. A., Tonkinson, J. L., and Yakubov, L. "Phosphorothioate oligodeoxynucleotides--anti-sense inhibitors of gene expression?". *Pharmacol Ther.* **52**(3): 365-84, **1991**. Review.

11.  Zon, G. and Geiser, T. G. "Phosphorothioate oligonucleotides: chemistry, purification, analysis, scale-up and future directions". *Anticancer Drug Des.* **6**(6): 539-68, **1991**. Review.

12.  Beaucage, S. L. and Iyer, R. P. "Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach". *Tetrahedron.* **48**(12): 2223-2311, **1992**. Review.

13.  Tidd, D. M. "Methylphosphonodiester/phosphodiester chimeric oligodeoxynucleotides". *Biochem Soc Trans.* **20**(4): 746-9, **1992**.

14.  Beaucage, S. L. and Iyer, R. P. "The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and their Applications". *Tetrahedron.* **49**(28): 6123-6194, **1993**. Review.

15.  Stirchak, E. P., Summerton, J. E., and Weller, D. E. *J. Org. Chem.* **52**: 4202-4206, **1987**.

16.  Stirchak, E. P., Summerton, J. E., and Weller, D. D. "Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside with oligomers with carbamate internucleoside linkages". *Nucl. Acids Res.* **15**: 6129-6140, **1989**.

71

17.   Pitha,  J.  and  Pitha,  P.  M.  *Biopolymers.*  **9**:  965,
**1970**.


18.   Pitha,  J.,  Pitha,  P.  M.,  and  Ts'o,  P.  O.  P.  *Biochem.*
5   *Biophys.  Acta.*  **204**:  39,  **1970**.


19.   Kropachev,  V.  A.,  et  al.  *Makromol.  Chem.  Suppl.*  **9**:
47-51,  **1985**.


10   20.   Takemoto,  K.  and  Inaki,  Y.  *Polym.  Mat.  Sci.  Eng.*  **58**:
250,  **1988**.


21.   Doel,  M.  T.,  Jones,  A.  S.,  and  Taylor,  N.  "An
Approach  to  the  Synthesis  of  Peptide  Analoges  of
15   Oligonucleotides  (Nucleopeptides)".  *Tettrahedron  Lett.*
**27**:  2285-2288,  **1969**.


22.   Nollet,  A.  J.  H.,  Huting,  C.  M.,  and  Pandit,  U.  K.
"Unconventional  Nucleotide  Analogues-I:  $N^9$-Purinyl  a-amino
20   acids".  *Tetrahedron.*  **25**:  5971-5981,  **1969**.


23.   Nollet,  A.  J.  H.  and  Pandit,  U.  K.  "Unconventional
Nucleotide  Analogues-II:  Synthesis  of  the  Adenyl  Analogue
of  Willardine".  *Tetrahedron.*  **25**:  5983-5887,  **1969**.
25
24.   Nollet,  A.  J.  H.  and  Pandit,  U.  K.  "Unconventional
Nucleotide Analogues-III: 4-(N1-Pyrimidyl)-2-Aminobutyric
Acids".  *Tetrahedron.*  **25**:  5989-5994,  **1969**.


30   25.   De  Koning,  H.  and  Pandit,  U.  K.  "Unconventional
Nucleotide  Analogues.  VI.:  Synthesis  of  Purinyl-  and
Pyrimidinyl-  Peptides".  *Rec.  Trav.  Chim.*  **91**:  1069-1081,
**1971**.

72

26.  Buttery, J. D., Jones, A. S., and Walker, R. T. "Synthetic  Analogues  of  Polynucleotides-XIII:  The Resolution  of  DL-b-(Thymin-1-yl)alanine  and Polymerization  of  the  b-(Thymin-1-yl)alanines". *Tetrahedron*. **31**: 73-75, **1975**.


27.  Lidak, M. Y., et al. *Khim. Getero. Sodinenii*. **11**: 1560-63, **1975**.


28.  Draminski, M. and Pitha, J. "Polypeptides Containing Adenine and Uracil Residues". *Makromol. Chem.* **179**: 2195-2200, **1978**.


29.  Olsuf'eva, E. N. and Shvachkin, Y. P. *Zh. Obs. Khim.* **49**: 1147-51, **1979**.


30.  Semiletov, Y. A., et al. *Zh. Obs. Khim.* **51**: 230-8, **1981**.


31.  Raukas, E., et al. *Stud. Biophys.* **89**: 187-195, **1982**.


32.  Lidak, M. Y., et al. *Khim. Getero. Soedinenii*. **3**: 402-5, **1983**.


33.  Cheikh, A. B. and Orgel, L. E. "Polymerization of Amino Acids containing Nucleotide Bases". *J. Mol. Evol.* **30**: 315-321, **1990**.


34.  Huang, S.-B., Nelson, J. S., and Weller, D. D. "Acyclic  Nucleic  Acid  Analogues:  Synthesis  and Oligomerization of gamma,4-Diamino-2-oxo-1(2H)-pyrimidinepentanoic Acid and delta,4-Diamino-2-oxo-1(2H)-pyrimidinehexanoic  acid". *J. Org. Chem.* **56**: 6007-6018, **1991**.

73

35. Weller, D. D., et al. "Molecular Modelling of Acyclic Polyamide Oligonucleotide Analogues". *J. Org. Chem.* **56**: 6000-6006, **1991**.

5  36. Nielsen, P. E., et al. "Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide". *Science.* **254**(5037): 1497-500, **1991**.

10  37. Egholm, M., et al. "Peptide Nucleic Acids (Pna) - Oligonucleotide Analogues With An Achiral Peptide Backbone". *Journal Of The American Chemical Society.* **114**(5): 1895-1897, **1992**.

15  38. Egholm, M., et al. "Recognition Of Guanine And Adenine In Dna By Cytosine And Thymine Containing Peptide Nucleic Acids (Pna)(1,2)". *Journal Of The American Chemical Society.* **114**(24): 9677-9678, **1992**.

20  39. Egholm, M., et al. "Peptide Nucleic Acids Containing Adenine Or Guanine Recognize Thymine And Cytosine In Complementary Dna Sequences". *Journal Of The Chemical Society-Chemical Communications.* (N9): 800-801, **1993**.

25  40. Egholm, M., et al. "PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules [see comments]". *Nature.* **365**(6446): 566-8, **1993**.

30  41. Dueholm, K. L., et al. "Peptide Nucleic Acid (Pna) With A Chiral Backbone Based On Alanine". *Bioorganic & Medicinal Chemistry Letters.* **4**(8): 1077-1080, **1994**.

42. Nielsen, P. E., Egholm, M., and Buchardt, O.
35  "Peptide Nucleic Acid (Pna) - A Dna Mimic With A Peptide Backbone". *Bioconjugate Chemistry.* **5**(1): 3-7, **1994**.

74

43.   Martin, F. H. and Castro, M. M. *Nucleic Acids Res.*
13: 8927, **1985**.


44.   Chollet, A. and Kawashima, E. *Nucleic Acids Res.* **16**:
5  305, **1988**.


45.   Inoue, H., Imura, A., and Ohtsuka, E. *Nucleic Acids
Res.* **13**: 7119, **1985**.


10  46.   Summerton, J. and Bartlett, P. A. *J. Mol. Biol.* **122**:
145, **1978**.


47.   Webb, T. R. and Matteucci, M. D. *J. Am. Chem. Soc.*
**108**: 2764, **1986**.
15
48.   Cowart, M., et al. *Biochemistry.* **28**: 1975, **1989**.


49.   Connolly, B. A. "Synthetic oligodeoxynucleotides
containing modified bases". *Methods Enzymol.* **211**(36):
20  36-53, **1992**. Review.


50.   Griffin, L. C., et al. "Recognition Of All 4 Base
Pairs Of Double-Helical Dna By Triple-Helix Formation -
Design    Of    Nonnatural    Deoxyribonucleosides    For
25  Pyrimidine.Purine Base Pair Binding". *Journal Of The
American Chemical Society.* **114**(21): 7976-7982, **1992**.


51.   Miller, P. S., et al. "Recognition Of A Guanine
Cytosine Base Pair By 8-Oxoadenine". *Biochemistry.*
30  **31**(29): 6788-6793, **1992**.


52.   Xu, Y. Z., Zheng, Q., and Swann, P. F. "Synthesis By
Post-Synthetic Substitution Of Oligomers Containing
Guanine Modified At The 6-Position With S-Derivatives,
35  N-Derivatives,    O-Derivatives". *Tetrahedron.*    **48**(9):
1729-1740, **1992**.

75

53. Xu, Y. Z., Zheng, Q. G., and Swann, P. F. "Synthesis Of Dna Containing Modified Bases By Postsynthetic Substitution - Synthesis Of Oligomers Containing 4-Substituted Thymine - O4-Alkylthymine,
5   5-Methylcytosine, N4-(Dimethylamino)-5-Methylcytosine, And 4-Thiothymine". *Journal Of Organic Chemistry*. **57**(14): 3839-3845, **1992**.

54. Kurfurst, R., et al. "Oligo-Alpha-
10  Deoxyribonucleotides With A Modified Nucleic Base And Covalently Linked To Reactive Agents". *Tetrahedron*. **49**(32): 6975-6990, **1993**.

55. Mohan, V., et al. "Molecular Recognition Of
15  Watson-Crick Base-Pair Reversals In Triple-Helix Formation - Use Of Nonnatural Oligonucleotide Bases". *Biopolymers*. **33**(9): 1317-1325, **1993**.

56. Sagi, J., et al. "Base-Modified
20  Oligodeoxynucleotides .1. Effect Of 5-Alkyl, 5-(1-Alkenyl) And 5-(1-Alkynyl) Substitution Of The Pyrimidines On Duplex Stability And Hydrophobicity". *Tetrahedron Letters*. **34**(13): 2191-2194, **1993**.

25  57. Sanghvi, Y. S., et al. "Antisense Oligodeoxynucleotides - Synthesis, Biophysical And Biological Evaluation Of Oligodeoxynucleotides Containing Modified Pyrimidines". *Nucleic Acids Research*. **21**(14): 3197-3203, **1993**.
30
58. Solomon, M. S. and Hopkins, P. B. "Chemical Synthesis And Characterization Of Duplex Dna Containing A New Base Pair - A Nondisruptive, Benzofused Pyrimidine Analog". *Journal Of Organic Chemistry*. **58**(8): 2232-2243,
35  **1993**.

76

59.  Grein, T., et al. "3-Deaza- And 7-Deazapurines - Duplex Stability Of Oligonucleotides Containing Modified Adenine Or Guanine Bases". *Bioorganic & Medicinal Chemistry Letters.* 4(8): 971-976, **1994.**

60.  Lin, P. K. and Brown, D. M. "Oligonucleotides containing degenerate bases. Synthesis and uses". *Methods Mol Biol.* 26(187): 187-206, **1994.** Review.

61.  Meyer, R. J. "Incorporation of modified bases into oligonucleotides". *Methods Mol Biol.* 26(73): 73-91, **1994.** Review.

62.  Reese, C. B. and Varaprasad, C. V. N. S. "Synthesis Of Some 2',3'-Didehydro-2',3'-Dideoxynucleosides Derived From Modified Pyrimidine Bases". *Journal Of The Chemical Society-Perkin Transactions 1.* (N2): 189-195, **1994.**

63.  Coppola, G. M. and Schuster, H. F. "Asymmetric Synthesis: Construction of Chiral Molecules Using Amino Acids." 1987 John Wiley & Sons. New York.

64.  O'Donnell, M. J. "a-Amino Acid Synthesis". *Tetrahedron.* 44: 5253-5614, **1988.** Review.

65.  Williams, R. M. "Synthesis of Optically Active a-Amino Acids." 1989 Pergamon Press. Oxford, U.K.

66.  Roberts, D. C. and Vellaccio, F. "Unusual Amino Acids in Peptide Synthesis." The Peptides. 1983 Academic Press Inc.

67.  Spatola, A. F. "Peptide Backbone Modifications: A Structure-Activity Analysis of Peptides containing Amide Bond Surrogates." Chemistry and Biochemistry of Amino Acids Peptides and Proteins. Weinstein ed. 1983 Marccel Dekker Inc. New York.

77

68. Gante, J. "Azapeptides". *Synthesis.* : 405-413, **1989**. Review.

69. Blaney, F. "Molecular Modelling In The Pharmaceutical Industry". *Chemistry & Industry.* (N23): 791-794, **1990**.

70. Laughton, C. A., et al. "Interaction Of Berenil With The Tyrt Dna Sequence Studied By Footprinting And Molecular Modelling - Implications For The Design Of Sequence-Specific Dna Recognition Agents". *Nucleic Acids Research.* **18**(15): 4479-4488, **1990**.

71. Sansom, C. E., et al. "Structural Studies On Bio-Active Compounds .14. Molecular Modelling Of The Interactions Between Pentamidine And Dna". *Anti-Cancer Drug Design.* **5**(3): 243-248, **1990**.

72. Crabbe, M. J. C. and Falkingbridge, S. S. "Molecular Modelling Of Hiv-1 Reverse Transcriptase Inhibitors". *Aids Research And Human Retroviruses.* **7**(3): 261-264, **1991**.

73. Letellier, R., et al. "Molecular Modelling Of 9-Aminoellipticine Interactions With Abasic Oligonucleotides". *Journal Of Biomolecular Structure & Dynamics.* **9**(3): 579-597, **1991**.

74. Jaroszewski, J. W., et al. "Towards rational design of antisense DNA: molecular modelling of phosphorothioate DNA analogues". *Anticancer Drug Des.* **7**(3): 253-62, **1992**.

75. Diana, G. D., et al. "Antipicornavirus Compounds - Use Of Rational Drug Design And Molecular Modelling". *Antiviral Chemistry & Chemotherapy.* **4**(1): 1-10, **1993**.

78

76. Ramsden, C. A. Quantitative Drug Design. Comprehensive Medicinal Chemistry. **4**: 33-58, 83-104, 105-124, 125-138, 413-498, **1990**, Pergammon Press, Oxford, U. K.

5

77. Martin, Y. C. "Computer-Assisted Rational Drug Design." in "Molecular Design and Modelling: Concepts and Applications", Langone, J.J., ed., Methods in Enzymology, 203:587-612, 1991 Academic Press Inc. San Diego.

10

78. Choplin, F. "Computers and the Medicinal Chemist." Quantitative Drug Design. Ramsden, C.A., ed., Comprehensive Medicinal Chemistry, 4:33-58, 1990 Pergammon Press. Oxford, U. K.

15

79. Loew, G. H. and Burt, S. K. "Quantum Mechanics and the Modelling of Drug Properties." Quantitative Drug Design. Ramsden, C.A., ed., Comprehensive Medicinal Chemistry, 4:105-124, 1990 Pergammon Press. Oxford, U. K.

20

80. Seibel, G. L. and Kollman, P. A. "Molecular Mechanics and the Modelling of Drug Structure." Quantitative Drug Design. Ramsden, C.A., ed., Comprehensive Medicinal Chemistry, 4:125-138, 1990

25 Pergammon Press. Oxford, U. K.

81. McCammon, J. A. "Dynamic Simulation and its Applications in Drug Research." Quantitative Drug Design. Ramsden, C.A., ed., Comprehensive Medicinal Chemistry,

30 4:139-152, 1990 Pergammon Press. Oxford, U. K.

82. Desjarlais, R., et al. *J. Med. Chem.* **31**: 722, **1988**.

83. Shoichet, B. K., Bodian, D. L., and Kuntz, I. D. *J.*

35 *Comput. Chem.* **13**: 380, **1992**.

79

84. Meng, E. C., Shoichet, B. K., and Kuntz, I. D. *J. Comut. Chem.* **13**: 505, **1992**.

85. Blaney, J. M. and Hansch, C. "Application of

5 Molecular Graphics to the Analysis of Macromolecular Structure." Quantitative Drug Design. Ramsden, C.A., ed., Comprehensive Medicinal Chemistry, 4:460-496, 1990 Pergammon Press. Oxford, U. K.

10 86. Marshall, G. R. and Naylor, C. B. "Use of Molecular Graphics for Structural Analysis of Small Molecules." Quantitative Drug Design. Ramsden, C.A., ed., Comprehensive Medicinal Chemistry, 4:432-458, 1990 Pergammon Press. Oxford, U. K.

15

87. Hansch, C. and et al. MEDCHEM[e]. Pomona College Medicinal Chemistry Laboratory, Seaver Chemistry Laboratory, Claremont, CA 91771.

20 88. Jurs, P. C., et al. "ADAPT[e]". *J. Chem. Inf. Comput. Sci.* **25**: 296, **1985**.

89. Rich, A. "DNA Comes in Many Forms". *Gene.* **135**(1-2): 99-109, **1993**. Review.

25

90. Fleisher, M. B., Mei, H., and Barton, J. K. "Metal Complexes which Target DNA Sites: Coupling Recognition to Reactivity." Nucleic Acids and Molecular Biology. Eckstein and Lilley ed. 1988 Springer-Verlag. Berlin,

30 FRG.

91. Chassagne, C. and Schwartz, K. "Mapping of mRNA isoforms with an oligonucleotide probe: exonuclease VII compared with endonucleases". *Nucleic Acids Res.* **20**(12):

35 3256, **1992**.

92.   Murray, V., et al. "Detection of polymorphisms using
thermal cycling with a single oligonucleotide on a DNA
sequencing gel". *Hum Mutat.* 2(2): 118-22, **1993**.


5   93.   Stahl, W. L., Eakin, T. J., and Baskin, D. G.
"Selection of oligonucleotide probes for detection of
mRNA isoforms". *J Histochem Cytochem.* 41(12): 1735-40,
**1993**.


10   94.   Stewart, J. M. and Young, J. D. "Solid Phase Peptide
Synthesis." 1984 Pierce Chemical Company. Rockford,
Illinois.


95.   Boado, R. J. and Pardridge, W. M. "Complete
15   protection of antisense oligonucleotides against serum
nuclease degradation by an avidin-biotin system".
*Bioconjug Chem.* 3(6): 519-23, **1992**.


96.   Bunnell, B. A., Askari, F. K., and Wilson, J. M.
20   "Targeted delivery of antisense oligonucleotides by
molecular conjugates". *Somat Cell Mol Genet.* 18(6):
559-69, **1992**.


97.   Chavany, C., et al. "Polyalkylcyanoacrylate
25   nanoparticles as polymeric carriers for antisense
oligonucleotides". *Pharm Res.* 9(4): 441-9, **1992**.


98.   Juliano, R. L. and Akhtar, S. "Liposomes as a drug
delivery system for antisense oligonucleotides".
30   *Antisense Res Dev.* 2(2): 165-76, **1992**.


99.   MacKellar, C., et al. "Synthesis and physical
properties of anti-HIV antisense oligonucleotides bearing
terminal lipophilic groups". *Nucleic Acids Res.* 20(13):
35   3411-7, **1992**.

81

100. Manoharan, M., et al. "Chemical modifications to improve uptake and bioavailability of antisense oligonucleotides". *Ann N Y Acad Sci.* **660**(306): 306-9, **1992**.

5

101. Ortigao, J. F., et al. "Antisense effect of oligodeoxynucleotides with inverted terminal internucleotidic linkages: a minimal modification protecting against nucleolytic degradation". *Antisense Res Dev.* **2**(2): 129-46, **1992**.

10

102. Ryte, A. S., et al. "Interaction of cholesterol-conjugated alkylating oligonucleotide derivatives with cellular biopolymers". *Febs Lett.* **299**(2): 124-6, **1992**.

15

103. Barry, E. L., Gesek, F. A., and Friedman, P. A. "Introduction of antisense oligonucleotides into cells by permeabilization with streptolysin O". *Biotechniques.* **15**(6): 1016-8, 1020, **1993**.

20

104. Capaccioli, S., et al. "Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum". *Biochem Biophys Res Commun.* **197**(2): 818-25, **1993**.

25

105. Clarenc, J. P., et al. "Delivery of antisense oligonucleotides by poly(L-lysine) conjugation and liposome encapsulation". *Anticancer Drug Des.* **8**(1): 81-94, **1993**. Review.

30

106. Gamper, H. B., et al. "Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides". *Nucleic Acids Res.* **21**(1): 145-50, **1993**.

35

107. Khan, I. M. and Coulson, J. M. "A novel method to stabilise antisense oligonucleotides against exonuclease degradation". *Nucleic Acids Res.* **21**(18): 4433, **1993**.

5  108. Leonetti, J. P., et al. "Cell delivery and mechanisms of action of antisense oligonucleotides". *Prog Nucleic Acid Res Mol Biol.* **44**(143): 143-66, **1993**. Review.

109. Zelphati, O., Zon, G., and Leserman, L. "Inhibition

10  of HIV-1 replication in cultured cells with antisense oligonucleotides encapsulated in immunoliposomes". *Antisense Res Dev.* **3**(4): 323-38, **1993**.

110. Maher(IIIrd), L. J. and Dolnick, B. J. *Biochem.*

15  *Biophys.* **253**: 214, **1987**.

111. Goodchild, J., et al. *Arch. Biochem. Biophys.* **263**: 401, **1988**.

20  112. Griffin, L. C. and Dervan, P. B. "Recognition of Thymine-Adenine Base Pairs by Guanine in a Pyrimidine Triple Helix Motif". *Science.* **245**: 967-971, **1989**.

113. Maher(IIIrd), L. J., Wold, B., and Dervan, P. B.

25  "Inhibition Of Dna Binding Proteins By Oligonucleotide-Directed Triple Helix Formation". *Science.* **245**(4919): 725-730, **1989**.

114. Moser, H. E. and Dervan, P. B. "Sequence-Specific

30  Cleavage of Double Helical DNA by Triple Helix Formation". *Science.* **238**: 645-650, **1987**.

115. Povsic, T. J. and et al. *J. Am. Chem. Soc.* **111**: 3059-3061, **1989**.

35

116. Lee, J. S. and et al. *Nucleic Acids Res.* **12**: 6603-6614, **1984**.

83

117. Cooney, M. and et al. *Science.* **241**: 456-459, **1988**.

118. Giovannangeli, C., et al. "Single-Stranded Dna As A Target For Triple-Helix Formation". *Journal Of The American Chemical Society.* **113**(20): 7775-7777, **1991**.

119. Beal, P. A. and Dervan, P. B. "Recognition Of Double Helical Dna By Alternate Strand Triple Helix Formation". *Journal Of The American Chemical Society.* **114**(13): 4976-4982, **1992**.

120. Giovannangeli, C., et al. "Triple-helix formation by oligonucleotides containing the three bases thymine, cytosine, and guanine". *Proc Natl Acad Sci U S A.* **89**(18): 8631-5, **1992**.

121. Helene, C., Thuong, N. T., and Harel, B. A. "Control of gene expression by triple helix-forming oligonucleotides. The antigene strategy". *Ann N Y Acad Sci.* **660**(27): 27-36, **1992**. Review.

122. Maher(IIIrd), L. J. "Dna Triple-Helix Formation - An Approach To Artificial Gene Repressors". *Bioessays.* **14**(12): 807-815, **1992**.

123. Mergny, J. L., et al. "Triple helix-specific ligands". *Science.* **256**(5064): 1681-4, **1992**.

124. Ono, A., Tso, P. O. P., and Kan, L. S. "Triplex Formation Of An Oligonucleotide Containing 2'-O-Methylpseudoisocytidine With A Dna Duplex At Neutral Ph". *Journal Of Organic Chemistry.* **57**(11): 3225-3230, **1992**.

125. Strobel, S. A. and Dervan, P. B. "Triple helix-mediated single-site enzymatic cleavage of megabase genomic DNA". *Methods Enzymol.* **216**(309): 309-21, **1992**.

126. Ts'o, P. O., et al. "Nonionic oligonucleotide analogs (Matagen) as anticodic agents in duplex and triplex formation". *Ann N Y Acad Sci.* **660**: 159-77, **1992**.

5  127. Asseline, U. and Thuong, N. T. "Oligonucleotides Tethered Via Nucleic Bases - A Potential New Set Of Compounds For Alternate Strand Triple-Helix Formation". *Tetrahedron Letters.* **34**(26): 4173-4176, **1993**.

10  128. Brossalina, E., Pascolo, E., and Toulme, J. J. "The binding of an antisense oligonucleotide to a hairpin structure via triplex formation inhibits chemical and biological reactions". *Nucleic Acids Res.* **21**(24): 5616-22, **1993**.

15

129. Davison, E. C. and Johnsson, K. "Triple Helix Binding Of Oligodeoxyribonucleotides Containing 8-Oxo-2'-Deoxyadenosine". *Nucleosides And Nucleotides.* **12**(2): 237-243, **1993**.

20

130. Han, H. and Dervan, P. B. "Sequence-specific recognition of double helical RNA and RNA.DNA by triple helix formation". *Proc Natl Acad Sci U S A.* **90**: 3806-10, **1993**.

25

131. Huang, C. Y., Cushman, C. D., and Miller, P. S. "Triplex Formation By An Oligonucleotide Containing N(4)-(3-Acetamidopropyl)Cytosine". *Journal Of Organic Chemistry.* **58**(19): 5048-5049, **1993**.

30

132. Jetter, M. C. and Hobbs, F. W. "7,8-Dihydro-8-Oxoadenine As A Replacement For Cytosine In The 3Rd Strand Of Triple Helices - Triplex Formation Without Hypochromicity". *Biochemistry.* **32**(13): 3249-3254,

35  **1993**.

85

133. Milligan, J. F., et al. "An anti-parallel triple helix motif with oligodeoxynucleotides containing 2'-deoxyguanosine and 7-deaza-2'-deoxyxanthosine". *Nucleic Acids Res.* **21**(2): 327-33, **1993**.

134. Sun, J. S. and Helene, C. "Oligonucleotide-Directed Triple-Helix Formation". *Current Opinion In Structural Biology.* **3**(3): 345-356, **1993**.

135. Tung, C. H., Breslauer, K. J., and Stein, S. "Polyamine-linked oligonucleotides for DNA triple helix formation". *Nucleic Acids Res.* **21**(23): 5489-94, **1993**.

136. Colocci, N. and Dervan, P. B. "Cooperative Binding Of 8-Mer Oligonucleotides Containing 5-(1-Propynyl)-2'-Deoxyuridine To Adjacent Dna Sites By Triple-Helix Formation". *Journal Of The American Chemical Society.* **116**(2): 785-786, **1994**.

137. Rao, T. S., et al. "Synthesis Of Triple Helix Forming Oligonucleotides With A Stretched Phosphodiester Backbone". *Nucleosides And Nucleotides.* **13**(1-3): 255-273, **1994**.

138. Helene, C. and Thuong, N. T. "Control of Gene Expression by Oligonucleotides Covalently Linked to Intercalating Agents". *Genome.* **31**(1): 413-421, **1989**. Review.

139. Sun, J. S., et al. "Sequence-Specific Intercalating Agents- Intercalation at Specific Sequences on Duplex DNA via Major Groove Recognition by Oligonucleotide Intercalator Conjugates". *Proc. Nat. Acad. Sci. USA.* **60**(N2): 157-160, **1989**.

140. Asseline, U. and Thuong, N. T. "Synthesis of Oligonucleotides covalently Linked to Intercalating

86

Agents and to Reactive Groups". *Nucleosides and Nucleotides*. **10**(1-3): 359-362, **1991**.

141. Baguley, B. C. "DNA intercalating anti-tumour
5  agents". *Anticancer Drug Des*. **6**(1): 1-35, **1991**. Review.

142. Bailly, C. and Henichart, J. P. "DNA recognition by intercalator-minor-groove binder hybrid molecules". *Bioconjug Chem*. **2**(6): 379-93, **1991**. Review.
10

143. Helene, C. "The anti-gene strategy: control of gene expression by triplex-forming-oligonucleotides". *Anticancer Drug Des*. **6**(6): 569-84, **1991**. Review.

15  144. Helene, C. and Thuong, N. T. "Design of bifunctional oligonucleotide intercalator conjugates as inhibitors of gene expression". *Nucleic Acids Symp Ser*. **1991**(24): 133-7, **1991**.

20  145. Sun, J. S., et al. "Triple-helix formation by alpha oligodeoxynucleotides and alpha oligodeoxynucleotide-intercalator conjugates". *Proc Natl Acad Sci U S A*. **88**: 6023-7, **1991**.

25  146. Pilch, D. S., et al. "Characterization of a triple helix-specific ligand. BePI (3-methoxy-7H-8-methyl-11-[(3'-amino)propylamino]-benzo[e]pyrido[4,3-b]indole) intercalates into both double-helical and triple-helical DNA". *J Mol Biol*. **232**(3): 926-46, **1993**.
30

147. Wilson, W. D., et al. "DNA triple-helix specific intercalators as antigene enhancers: unfused aromatic cations". *Biochemistry*. **32**(40): 10614-21, **1993**.

35  148. Mouscadet, J. F., et al. "Triple helix formation with short oligonucleotide-intercalator conjugates

87

matching the HIV-1 U3 LTR end sequence". *Biochemistry*.
**33**(14): 4187-96, **1994**.

149. Le Doan, T., et al. *Nucleic Acids Res.* **15**: 7749,
5   **1987**.

150. Praseuth, D., et al. *Proc Natl. Acad. Sci. USA.* **85**:
1349, **1988**.

10  151. Vlassov, V. V., et al. *Gene.* **72**: 313, **1988**.

152. Fedorova, O. S., et al. *FEBS Lett.* **228**: 273, **1988**.

153. Greenfield, L., Bloch, W., and Moreland, M.
15  "Thiol-containing cross-linking agent with enhanced
steric hindrance". *Bioconjug Chem.* **1**(6): 400-10, **1990**.

154. Perrouault, L., et al. "Sequence-specific artificial
photo-induced endonucleases based on triple helix-forming
20  oligonucleotides". *Nature.* **344**(6264): 358-60, **1990**.

155. Jackson, C., et al.
"N2,N4,N6-tri(hydroxymethyl)-N2,N4,N6-trimethylmelamine
(trimelamol) is an efficient DNA cross-linking agent in
25  vitro". *Biochem Pharmacol.* **42**(11): 2091-7, **1991**.

156. Le Doan, T., et al. "Recognition and photo-induced
cleavage and cross-linking of nucleic acids by
oligonucleotides covalently linked to ellipticine".
30  *Antisense Res Dev.* **1**(1): 43-54, **1991**.

157. Giovannangeli, C., Thuong, N. T., and Helene, C.
"Oligodeoxynucleotide-directed photo-induced
cross-linking of HIV proviral DNA via triple-helix
35  formation". *Nucleic Acids Res.* **20**(16): 4275-81, **1992**.

88

158. Miller, P. S. "Preparation of psoralen-derivatized oligodeoxyribonucleoside methylphosphonates". *Methods Enzymol.* **211**(54): 54-64, **1992**. Review.

5   159. Havre, P. A., et al. "Targeted mutagenesis of DNA using triple helix-forming oligonucleotides linked to psoralen". *Proc Natl Acad Sci U S A.* **90**(16): 7879-83, **1993**.

10  160. Rajagopalan, K., et al. "Synthesis and application of bidentate photoaffinity cross-linking reagents. Nucleotide photoaffinity probes with two photoactive groups". *J Biol Chem.* **268**(19): 14230-8, **1993**.

15  161. Boutorine, A. S., et al. "Rapid routes of synthesis of chemically reactive and highly radioactively labeled alpha- and beta-oligonucleotide derivatives for in vivo studies". *Bioconjug Chem.* **1**(5): 350-6, **1990**.

20  162. Farquharson, M., Harvie, R., and McNicol, A. M. "Detection of messenger RNA using a digoxigenin end labelled oligodeoxynucleotide probe". *J Clin Pathol.* **43**(5): 424-8, **1990**.

25  163. Haralambidis, J., et al. "The preparation of polyamide-oligonucleotide probes containing multiple non-radioactive labels". *Nucleic Acids Res.* **18**(3): 501-5, **1990**.

30  164. Strobel, O. K., et al. "Preparation and characterization of spin-labeled oligonucleotides for DNA hybridization". *Bioconjug Chem.* **2**(2): 89-95, **1991**.

    165. Nelson, P. S., Kent, M., and Muthini, S.
35  "Oligonucleotide labeling methods. 3. Direct labeling of oligonucleotides employing a novel, non-nucleosidic,

89

2-aminobutyl-1,3-propanediol backbone". *Nucleic Acids Res.* **20**(23): 6253-9, **1992**.

166. Durrant, I. and Chadwick, P. M. "Hybridization of
5 fluorescein-labeled oligonucleotide probes and enhanced chemiluminescence detection". *Methods Mol Biol.* **28**(141): 141-8, **1994**. Review.

167. Brossalina, E., Demchenko, E., and Vlassov, V.
10 "Sequence specific alkylation of dsDNA by 2-chloroethylamine derivatives of purine oligonucleotides". *Nucleic Acids Symp Ser.* **1991**(24): 262, **1991**.

15 168. Brossalina, E. B., et al. "Sequence-specific alkylation of dsDNA with derivatives of pyrimidine oligonucleotides conjugated to 2-chloroethylamine groups". *Antisense Res Dev.* **1**(3): 229-42, **1991**.

20 169. Gourdie, T. A., et al. "Synthesis and evaluation of DNA-targeted spatially separated bis(aniline mustards) as potential alkylating agents with enhanced DNA cross-linking capability". *J Med Chem.* **34**(1): 240-8, **1991**.
25

170. Gravatt, G. L., et al. "DNA-directed alkylating agents. 4. 4-anilinoquinoline-based minor groove directed aniline mustards". *J Med Chem.* **34**(5): 1552-60, **1991**.

30 171. Prakash, A. S., et al. "Synthesis and anti-tumour activity of the spatially-separated mustard bis-N,N'-[3-(N-(2-chloroethyl)-N-ethyl)amino-5-[N,N-dim e t h y l a m i n o ) m e t h y 1)-aminophenyl]-1,4-benzenedicarboxamide, which alkylates
35 DNA exclusively at adenines in the minor groove". *Anticancer Drug Des.* **6**(3): 195-206, **1991**.

90

172. Zarytova, V. F., et al. "[Sequence-specific modification of nucleic acids by oligonucleotide derivative containing alkylating groups in the C-5-position of deoxyuridine]". *Bioorg Khim*. **18**(5): 640-5, **1992**.

173. Berger, N. A. "Alkylating agents". *Cancer Chemother Biol Response Modif*. **14**(26): 26-36, **1993**. Review.

174. Lee, M., et al. "Design, synthesis, and biological evaluation of DNA sequence and minor groove selective alkylating agents". *Anticancer Drug Des*. **8**(3): 173-92, **1993**.

175. Yamamoto, K., Sugiyama, H., and Kawanishi, S. "Concerted DNA recognition and novel site-specific alkylation by duocarmycin A with distamycin A". *Biochemistry*. **32**(4): 1059-66, **1993**.

176. Zhang, Y., et al. "Groove- and sequence-selective alkylation of DNA by sulfonate esters tethered to lexitropsins". *Biochemistry*. **32**(31): 7954-65, **1993**.

177. Lee, M., et al. "In vitro photoinduced cytotoxicity and DNA binding properties of psoralen and coumarin conjugates of netropsin analogues: DNA sequence-directed alkylation and cross-link formation". *J Med Chem*. **37**(8): 1208-13, **1994**.

178. Le Doan, T., et al. *Bioconjugate Chem*. **1**: 108, **1990**.

179. Bhan, P. and Miller, P. S. "Photo-cross-linking of psoralen-derivatized oligonucleoside methylphosphonates to single-stranded DNA". *Bioconjug Chem*. **1**(1): 82-8, **1990**.

91

180. Chatterjee, M. and Rokita, S. E. *J. Am. Chem. Soc.* **112**: 9387, **1990**.

181. Celander, D. W. and Cech, T. R. "Iron(II)-ethylenediaminetetraacetic acid catalyzed cleavage of RNA and DNA oligonucleotides: similar reactivity toward single- and double-stranded forms". *Biochemistry.* **29**(6): 1355-61, **1990**.

182. Sigman, D. S. and Chen, C. H. "Chemical nucleases: new reagents in molecular biology". *Annu Rev Biochem.* **59**(207): 207-36, **1990**. Review.

183. Nagai, K. and Hecht, S. M. "Site-specific DNA cleavage by antisense oligonucleotides covalently linked to phenazine di-N-oxide". *J Biol Chem.* **266**(35): 23994-4002, **1991**.

184. Jayasena, S. D. and Johnston, B. H. "Site-specific cleavage of the transactivation response site of human immunodeficiency virus RNA with a tat-based chemical nuclease". *Proc Natl Acad Sci U S A.* **89**(8): 3526-30, **1992**.

185. Podhajska, A. J., Kim, S. C., and Szybalski, W. "Conferring new specificities on restriction enzymes: cleavage at any predetermined site by combining adapter oligodeoxynucleotide and class-IIS enzyme". *Methods Enzymol.* **216**(303): 303-9, **1992**.

186. Casas, C., Lacey, C. J., and Meunier, B. "Preparation of hybrid "DNA cleaver-oligonucleotide" molecules based on a metallotris(methylpyridiniumyl)porphyrin motif". *Bioconjug Chem.* **4**(5): 366-71, **1993**.

92

187. Chen, C. B., Gorin, M. B., and Sigman, D. S. "Sequence-specific scission of DNA by the chemical nuclease activity of 1,10-phenanthroline-copper(I) targeted by RNA". *Proc Natl Acad Sci U S A.* 90(9):
5   4206-10, **1993**.


188. Huber, P. W. "Chemical nucleases: their use in studying RNA structure and RNA-protein interactions". *Faseb J.* 7(14): 1367-75, **1993**. Review.
10

189. Kappen, L. S. and Goldberg, I. H. "DNA conformation-induced activation of an enediyne for site-specific cleavage". *Science.* 261(5126): 1319-21, **1993**.
15

190. Sigman, D. S., Chen, C. H., and Gorin, M. B. "Sequence-specific scission of DNA by RNAs linked to a chemical nuclease". *Nature.* 363(6428): 474-5, **1993**.


20  191. Zarytova, V. F., Sergeyev, D. S., and Godovikova, T. S. "Synthesis of bleomycin A5 oligonucleotide derivatives and site-specific cleavage of the DNA target". *Bioconjug Chem.* 4(3): 189-93, **1993**.


25  192. Shimizu, M., Inoue, H., and Ohtsuka, E. "Detailed study of sequence-specific DNA cleavage of triplex-forming oligonucleotides linked to 1,10-phenanthroline". *Biochemistry.* 33(2): 606-13, **1994**.


30  193. Froncois, et al. *Proc. Natl. Acad. Sci. USA.* 86: 9702, **1989**.


194. Froncois, et al. *J. Biol. Chem.* 264: 5891, **1989**.


35  195. Le Doan, T., et al. *Nucleic Acids Res.* 15: 8643, **1987**.

196. Corey, D. R. and Schultz, P. G. *Science.* **238**: 1401, **1987**.

197. Zuckermann, R. N., Corey, D. R., and Schultz, P. G. *J. Am. Chem. Soc.* **110**: 1614, **1988**.

198. Pei, D., Corey, D. R., and Schultz, P. G. "Site-specific cleavage of duplex DNA by a semisynthetic nuclease via triple-helix formation". *Proc Natl Acad Sci U S A.* **87**(24): 9858-62, **1990**.

199. Haseloff, J. and Gerlach, W. L. *Nature.* **334**: 585, **1988**.

200. Jeffries, A. C. and Symons, R. H. *Nucleic Acids Res.* **17**: 1371, **1989**.

201. Van, A. R. and Hecht, S. M. "A ribozyme model: site-specific cleavage of an RNA substrate by Mn2+". *Adv Inorg Biochem.* **9**(1): 1-40, **1994**. Review.

202. Puglisi, J. D. and Tinoco, I. J. "Absorbance melting curves of RNA". *Methods Enzymol.* **180**(304): 304-25, **1989**. Review.

203. Jaeger, J. A., SantaLucia, J. J., and Tinoco, I. J. "Determination of RNA structure and thermodynamics". *Annu Rev Biochem.* **62**(255): 255-87, **1993**. Review.

204. Breslauer, K. J. "Extracting thermodynamic data from equilibrium melting curves for oligonucleotide order-disorder transitions". *Methods Mol Biol.* **26**(347): 347-72, **1994**. Review.

205. Nielsen, P. E. "Chemical and photochemical probing of DNA complexes". *J Mol Recognit.* **3**(1): 1-25, **1990**. Review.

94

206. Zhong, W. X., et al. "DNA solution conformation via infrared circular dichroism: experimental and theoretical results for B-family polymers". *Biochemistry*. 29(32): 7485-91, **1990**.

5

207. Gray, D. M., Ratliff, R. L., and Vaughan, M. R. "Circular dichroism spectroscopy of DNA". *Methods Enzymol*. 211(389): 389-406, **1992**. Review.

10  208. Lilley, D. M., Bhattacharyya, A., and McAteer, S. "Gel electrophoresis and the structure of RNA molecules". *Biotechnol Genet Eng Rev*. 10(379): 379-401, **1992**. Review.

209. Ussery, D. W., Hoepfner, R. W., and Sinden, R. R.
15  "Probing DNA structure with psoralen in vitro". *Methods Enzymol*. 212(242): 242-62, **1992**. Review.

210. Freedman, T. B. and Nafie, L. A. "Infrared circular dichroism". *Methods Enzymol*. 226(306): 306-19, **1993**.
20  Review.

211. Jones, C. "Applications of nuclear magnetic resonance, circular dichroism and fluorescence spectroscopy to the characterization of biological
25  products". *Biologicals*. 21(2): 119-24, **1993**.

212. Sylvers, L. A. and Wower, J. "Nucleic acid-incorporated azidonucleotides: probes for studying the interaction of RNA or DNA with proteins and other
30  nucleic acids". *Bioconjug Chem*. 4(6): 411-8, **1993**.

213. Thomson, A. J., Cheesman, M. R., and George, S. J. "Variable-temperature magnetic circular dichroism". *Methods Enzymol*. 226(199): 199-232, **1993**. Review.

35
214. Galas, D. J. and Schmitz, A. "Footprinting". *Nucleic Acids Res*. 5: 3157, **1978**.

215. Tullius, T. D., et al. "Hydroxy Radical Footprinting: A High Resolution Method for Mapping Protein-DNA Contacts." Recombinant DNA (Part F). Wu ed. 1987 Academic Press Inc. New York, NY.

216. Dixon, W. J., et al. "Hydroxyl radical footprinting". *Methods Enzymol*. **208**(380): 380-413, **1991**. Review.

217. Tullius, T. D. "DNA footprinting with the hydroxyl radical". *Free Radic Res Commun*. **2**(521): 521-9, **1991**. Review.

218. Hayes, J. J., Kam, L., and Tullius, T. D. "Footprinting Protein-DNA Complexes with Gamma-rays." Oxygen Radicals in Biological Systems (Part B: Oxygen Radicals and Antioxidants. WU ed. 1990 Academic Press Inc. New York, NY.

219. Duval, V. G., Thuong, N. T., and Helene, C. "Specific inhibition of transcription by triple helix-forming oligonucleotides". *Proc Natl Acad Sci U S A*. **89**(2): 504-8, **1992**.

220. Metzger, W., et al. "Hydroxyl radical footprint analysis of human immunodeficiency virus reverse transcriptase-template.primer complexes". *Proc Natl Acad Sci U S A*. **90**(13): 5909-13, **1993**.

221. Mah, S. C., Townsend, C. A., and Tullius, T. D. "Hydroxyl radical footprinting of calicheamicin. Relationship of DNA binding to cleavage". *Biochemistry*. **33**(2): 614-21, **1994**.

222. "NACB 13th annual symposium: diagnostic applications of nucleic acid probes in the clinical laboratory.

96

Toronto, Canada, June 2-3, 1989. Proceedings". *Clin Biochem.* **23**(4): 253-340, **1990**. Review.

223. Barker, R. J. "DNA probe diagnosis of parasitic
5 infections". *Exp Parasitol.* **70**(4): 494-9, **1990**.

224. Gillespie, D. "The magic and challenge of DNA probes as diagnostic reagents". *Vet Microbiol.* **24**(3-4): 217-33, **1990**. Review.
10

225. Kohne, D. E. "The use of DNA probes to detect and identify microorganisms". *Adv Exp Med Biol.* **263**(11): 11-35, **1990**. Review.

15 226. Char, S. and Farthing, M. J. "DNA probes for diagnosis of intestinal infection". *Gut.* **32**(1): 1-3, **1991**.

227. Pfaller, M. A. "Diagnostic applications of DNA
20 probes". *Infect Control Hosp Epidemiol.* **12**(2): 103-10, **1991**. Review.

228. Peters, C., et al. "Individual-specific DNA fingerprinting in man using the oligonucleotide probe
25 (GTG)5/(CAC)5". *Eur J Clin Chem Clin Biochem.* **29**(5): 321-5, **1991**.

229. Wetmur, J. G. "DNA probes: applications of the principles of nucleic acid hybridization". *Crit Rev*
30 *Biochem Mol Biol.* **26**(3-4): 227-59, **1991**. Review.

230. Narayanan, S. "Overview of principles and current uses of DNA probes in clinical and laboratory medicine". *Ann Clin Lab Sci.* **22**(6): 353-76, **1992**. Review.
35

231. Rotbart, H. A. "DNA probes for viral diagnosis". *Adv Exp Med Biol.* **312**(201): 201-9, **1992**. Review.

97

232. Krawczak, M., et al. "Paternity testing with oligonucleotide multilocus probe (CAC)5/(GTG)5: a multicenter study". *Forensic Sci Int.* **59**(2): 101-17, **1993**.

233. Utz, E. D., et al. "Detection of human tRNAs with antisense oligonucleotides". *Anal Biochem.* **216**(1): 110-7, **1994**.

234. Agrawal, S. and Zamecnik, P. C. "Site specific functionalization of oligonucleotides for attaching two different reporter groups". *Nucleic Acids Res.* **18**(18): 5419-23, **1990**.

235. Misiura, K., et al. "Biotinyl and phosphotyrosinyl phosphoramidite derivatives useful in the incorporation of multiple reporter groups on synthetic oligonucleotides". *Nucleic Acids Res.* **18**(15): 4345-54, **1990**.

236. Tang, J. Y. and Agrawal, S. "Incorporation of multiple reporter groups on synthetic oligonucleotides". *Nucleic Acids Res.* **18**(21): 6461, **1990**.

237. Dirks, R. W., et al. "3'-end fluorochromized and haptenized oligonucleotides as in situ hybridization probes for multiple, simultaneous RNA detection". *Exp Cell Res.* **194**(2): 310-5, **1991**.

238. Murakami, A., et al. "Fluorescent-labeled oligonucleotide probes: detection of hybrid formation in solution by fluorescence polarization spectroscopy". *Nucleic Acids Res.* **19**(15): 4097-102, **1991**.

239. Will, D. W., Pritchard, C. E., and Brown, T. "The synthesis of oligonucleotides that contain

98

2,4-dinitrophenyl reporter groups". *Carbohydr Res.*
**216**(315): 315-22, **1991**.

240. Grzybowski, J., et al. "Synthesis and
5　antibody-mediated detection of oligonucleotides
containing multiple 2,4-dinitrophenyl reporter groups".
*Nucleic Acids Res.* **21**(8): 1705-12, **1993**.

241. Agrawal, S. "Functionalization of oligonucleotides
10　with amino groups and attachment of amino specific
reporter groups". *Methods Mol Biol.* **26**(93): 93-120, **1994**.
Review.

242. Fidanza, J. A., Ozaki, H., and McLaughlin, L. W.
15　"Functionalization of oligonucleotides by the
incorporation of thio-specific reporter groups". *Methods
Mol Biol.* **26**(121): 121-43, **1994**. Review.

243. Balaguer, P., et al. "Detection of single base
20　substitutions in polynucleotides by capture with
immobilized oligonucleotides". *Mol Cell Probes.* **7**(2):
155-9, **1993**.

244. Kawasaki, E., Saiki, R., and Erlich, H. "Genetic
25　analysis using polymerase chain reaction-amplified DNA
and immobilized oligonucleotide probes: reverse dot-blot
typing". *Methods Enzymol.* **218**(369): 369-81, **1993**. Review.

245. Kawasaki, E. S. and Chehab, F. F. "Analysis of gene
30　sequences by hybridization of PCR-amplified DNA to
covalently bound oligonucleotide probes. The reverse dot
blot method". *Methods Mol Biol.* **28**(225): 225-36, **1994**.
Review.

35　246. Boiziau, C., et al. "Ribonuclease H-mediated
inhibition of translation and reverse transcription by

99

antisense oligodeoxynucleotides". *Biochem Soc Trans.* **20**(4): 764-7, **1992**.

247. Calabretta, B., et al. "Prospects for gene-directed therapy with antisense oligodeoxynucleotides". *Cancer Treat Rev.* **19**(2): 169-79, **1993**. Review.

248. Cumin, F., et al. "Modulation of human prorenin gene expression by antisense oligonucleotides in transfected CHO cells". *Eur J Biochem.* **212**(2): 347-54, **1993**.

249. Nagel, K. M., Holstad, S. G., and Isenberg, K. E. "Oligonucleotide pharmacotherapy: an antigene strategy". *Pharmacotherapy.* **13**(3): 177-88, **1993**. Review.

250. Schreier, H. "The new frontier: gene and oligonucleotide therapy". *Pharm Acta Helv.* **68**(3): 145-59, **1994**. Review.

251. Inoue, M. *Gene.* **72**: 25, **1988**.

252. Winkler, M. E., et al. *Proc. Natl. Acad. Sci. USA.* **79**: 2181, **1982**.

253. Crooke, R. M. "In vitro toxicology and pharmacokinetics of antisense oligonucleotides". *Anticancer Drug Des.* **6**(6): 609-46, **1991**. Review.

254. Mirabelli, C. K., et al. "In vitro and in vivo pharmacologic activities of antisense oligonucleotides". *Anticancer Drug Des.* **6**(6): 647-61, **1991**.

255. Whitesell, L., Rosolen, A., and Neckers, L. M. "In vivo modulation of N-myc expression by continuous perfusion with an antisense oligonucleotide". *Antisense Res Dev.* **1**(4): 343-50, **1991**.

100

256. Bordier, B., et al. "In vitro effect of antisense oligonucleotides on human immunodeficiency virus type 1 reverse transcription". *Nucleic Acids Res.* **20**(22): 5999-6006, **1992**.

257. Hanvey, J. C., et al. "Antisense and antigene properties of peptide nucleic acids". *Science.* **258**(5087): 1481-5, **1992**.

258. Lisziewicz, J., et al. "Specific inhibition of human immunodeficiency virus type 1 replication by antisense oligonucleotides: an in vitro model for treatment". *Proc Natl Acad Sci U S A.* **89**(23): 11209-13, **1992**.

259. Woolf, T. M., Melton, D. A., and Jennings, C. G. "Specificity of antisense oligonucleotides in vivo". *Proc Natl Acad Sci U S A.* **89**(16): 7305-9, **1992**.

260. Wu, G. Y. and Wu, C. H. "Specific inhibition of hepatitis B viral gene expression in vitro by targeted antisense oligonucleotides". *J Biol Chem.* **267**(18): 12436-9, **1992**.

261. Nielsen, P. E., et al. "Peptide nucleic acids (PNAs): potential antisense and anti-gene agents". *Anticancer Drug Des.* **8**(1): 53-63, **1993**.

262. Offensperger, W. B., et al. "In vivo inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides". *Embo J.* **12**(3): 1257-62, **1993**.

263. Praseuth, D., et al. "Unexpected effect of an anti-human immunodeficiency virus intermolecular triplex-forming oligonucleotide in an in vitro transcription system due to RNase H-induced cleavage of

101

the RNA transcript". *Antisense Res Dev.* 3(1): 33-44, **1993**.

264. Volkmann, S., Dannull, J., and Moelling, K. "The polypurine tract, PPT, of HIV as target for antisense and triple-helix-forming oligonucleotides". *Biochimie.* 75(1-2): 71-8, **1993**.

265. Bennett, C. F., et al. "Inhibition of endothelial cell adhesion molecule expression with antisense oligonucleotides". *J Immunol.* 152(7): 3530-40, **1994**.

266. Alama, A., et al. "The use of antisense oligodeoxynucleotides (aODNs) for the therapy of cancer". *Drugs Exp Clin Res.* 17(12): 575-9, **1991**.

267. Cohen, J. S. "Antisense oligodeoxynucleotides as antiviral agents". *Antiviral Res.* 16(2): 121-33, ·**1991**. Review.

268. Agrawal, S. "Antisense Oligonucleotides as Antiviral Agents". *TIBTECH.* 10: 152-158, **1992**. Review.

269. Coulson, J. and Malcolm, A. D. "Antisense oligonucleotides as antiviral agents". *Ann N Y Acad Sci.* 660(339): 339-41, **1992**.

270. Cantin, E. M. and Woolf, T. M. "Antisense oligonucleotides as antiviral agents: prospects and problems". *Trends Microbiol.* 1(7): 270-6, **1993**. Review.

271. Kabanov, A. V., et al. *FEBS Lett.* 259: 327, **1990**.

272. Shea, R. G., Masters, J. C., and Bischofberger, N. *Nucleic Acids Res.* 18: 3777, **1990**.

102

273. Boutorin, A., et al. "Synthesis of Alkylating Oligonucleotide Derivatives Containing Cholesterol or Phenezeniumresidues at 3'-terminusand Their Interaction with mammalian Cells". *FEBS Lett.* **254**: 129, **1989**.

274. Oberhauser, B. and Wagner, E. "Effective Incorporation of 2'-O-methyl oligoribonucleotides into Liposomes and Enhanced Cell Association through Modfication with Thiocholesterol". *Nucleic Acids Res.* **20**: 533, **1992**.

275. SGI. IRIS Work-Station. Silicon Graphics Inc., Mountain View, CA.

276. SYBYL. SYBYL* Molecular Modelling Package. V 5.4/6.0, **1992**, TRIPOS Associates Inc., St. Louis, Missouri 63144-2913.

277. Arnott, S. and Hukins, D. W. L. "Crystal-structure of ds-DNA". *Biochem. Biophys. Res. Commun.* **47**: 1504, **1972**.

278. March, J. "Advanced Organic Chemistry: Reactions, Mechanisms and Structure." 1992 John Wiley & Sons. New York, NY.

279. Larock, R. C. "Comprehensive Organic Transformations: A Guide to Functional Group Preparations." 1989 VCH Publishers, Inc. New York.

280. Hooz, J. and Gilani, S. S. H. "A Rapid, Mild Procedure for the Preparation of Alkyl Chlorides and Bromides". *Canadian J. Chem.* **46**: 86-87, **1968**.

281. Bajgrowicz, J. A., et al. "Organocuperates Mediated Carbon-Carbon Bond Formation in gamma position of

103

alpha-amino esters without racemization". *Tetrahedron Lett.* **25**: 2231-2234, **1984**.

282. Jost, K. and Rudinger, J. "Amino Acids and Peptides. LXXIV.: Derivatives of L-Cystathione suitable for Peptide Synthesis". *Coll. Czech. Chem. Commun.* **32**: 2485-2490, **1967**.

283. Natelson, S. and Natelson, E. A. "Preparation of D-, DL-, and L-Homoserine Lactone from Methionine". *Microchemical Journal.* **40**: 226-232, **1989**.

284. Valerio, R. M., Alewood, P. F., and Johns, R. B. "Synthesis of Optically Active 2-(tert-Butyloxycarbonylamino)-4-dialkoxyphosphoryl-butanoate Protected Isosteres of O-phosphonoserine for Peptide Synthesis". *Synthesis.* : 786-789, **1988**.

285. Carpino, L. A. "1-Hydroxy-7-azabenzotriazole. An efficient Peptide Coupling Additive". *J. Am. Chem. Soc.* **115**: 4397-98, **1993**.

104

<u>Claims</u>

1.  A composition useful in targeting a nucleic acid
comprising a stereochemically-selected population of
5  peptide-based nucleic acid mimics (PENAMs) each member of
which comprises a sequence of at least about 4 NuAA
monomers,
        wherein at least one of said PENAMs is a
homomorphically-preferred PENAM comprising a D-chiral
10  center or a quasi-chiral and wherein said
homomorphically-preferred PENAM makes up at least about
10% of the stereochemically-selected population of
PENAMs.

15      2.  A PENAM composition of claim 1, wherein said
NuAA monomers are each of the following formula:
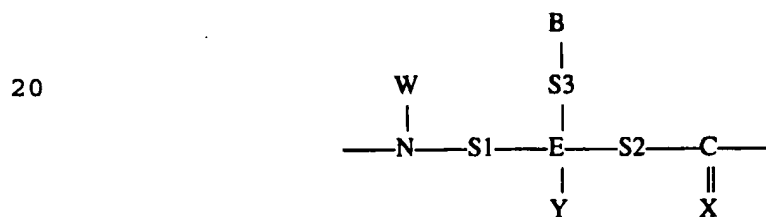
$$
\begin{array}{ccccc}
 & & B & & \\
 & & | & & \\
W & & S3 & & \\
| & & | & & \\
\text{---N---S1---E---S2---C---} \\
 & & | & & \| \\
 & & Y & & X \\
\end{array}
$$

25

wherein:
        E is carbon (C) or nitrogen (N);
        W is hydrogen or a spacer group;
        Y is hydrogen or a spacer group (when E is carbon),
30  or Y is a lone pair of electrons (when E is nitrogen);
        S1 is a bond or a first spacer group;
        S2 is a bond or a second spacer group;
        S3 is a bond or a third spacer group;
        X is oxygen (O) or sulfur (S);
35      B is a base segment comprising a nucleotidic base or
        an analog thereof; and
        N is nitrogen.

105

3. A PENAM composition of claim 2, wherein the NuAA monomers in said homomorphically-preferred PENAM are joined directly to each other via peptide bonds and at least one of S1 and S2 in each of the adjacent NuAA 5 monomers is a spacer group.

4. A PENAM composition of claim 2, wherein the NuAA monomers in said homomorphically-preferred PENAM are joined via intervening aminoacyl residues and S2 in each 10 of the NuAA monomers is a bond.

5. A PENAM composition of claim 2, wherein said homomorphically-preferred PENAM comprises a D-chiral center and said D-chiral center is located within a NuAA 15 monomer at position E wherein E is carbon.

6. A PENAM composition of claim 2, wherein said homomorphically-preferred PENAM comprises a D-chiral center and said D-chiral center is located between two 20 NuAA monomers at a chiral carbon in an intervening amino acid residue.

7. A PENAM composition of claim 2, wherein at least about 50% of the chiral centers in said homomorphically- 25 preferred PENAM are D-chiral centers.

8. A PENAM composition of claim 2, wherein about 50% of the chiral centers in said homomorphically- preferred PENAM are D-chiral centers, and 50% of the 30 chiral centers are L-chiral centers, and wherein at least about 80% of L-chiral centers are flanked by D-chiral centers.

9. A PENAM composition of claim 2, wherein at least 35 about 80% of the chiral centers in said homomorphically- preferred PENAM are D-chiral centers.

10.    A PENAM composition of claim 2, wherein at least one of S1 and S2 is a spacer group having a backbone of two to three atoms, S3 is a spacer group having a backbone of two to three atoms, W is hydrogen,

5    E is carbon, and Y is hydrogen.

11.    A PENAM composition of claim 2, wherein said homomorphically-preferred PENAM comprises a quasi-chiral center and said quasi-chiral center is located within a

10    NuAA monomer at position E wherein E is nitrogen.

12.    A PENAM composition of claim 2, wherein said homomorphically-preferred PENAM comprises a quasi-chiral center and said quasi-chiral center is located between

15    two NuAA monomers in an intervening aza-amino acid residue.

13.    A PENAM composition of claim 2, wherein all of the NuAA monomers in said homomorphically-preferred PENAM

20    comprise quasi-chiral centers.

14.    A PENAM composition of claim 2, wherein said homomorphically-preferred PENAM further comprises a target modifying group.

25

15.    A PENAM composition of claim 14, wherein said target modifying group is located within a NuAA monomer.

16.    A PENAM composition of claim 14, wherein said

30    target modifying group is located between NuAA monomers or outside of a string of NuAA monomers.

17.    A free NuAA monomer that is a precursor of a
NuAA monomeric subunit comprising a quasi-chiral center
wherein said NuAA monomeric subunit comprising a quasi-
chiral center is of the following formula:

```
                          B
                          |
             W            S3
             |            |
        ——N——S1——E——S2——C——
                      |       ‖
                      Y       X
```

wherein:
        E is nitrogen (N);
        W is hydrogen or a spacer group;
        Y is hydrogen or a spacer group (when E is carbon),
or Y is a lone pair of electrons (when E is nitrogen);
        S1 is a bond or a first spacer group;
        S2 is a bond or a second spacer group;
        S3 is a bond or a third spacer group;
        X is oxygen (O) or sulfur (S);
        B is a base segment comprising a nucleotidic base or
        an analog thereof; and
        N is nitrogen;
        wherein said free NuAA monomer is of the same
formula except that the N-terminus is in the form of an
amine group or a protected amine group and the C-terminus
is in the form of a carboxyl group or a protected
carboxyl group.

108

18.    A free NuAA monomer that is a precursor of a NuAA monomeric subunit comprising a D-chiral center wherein said NuAA monomeric subunit comprising a D-chiral center is of the following formula:

```
                          B
                          |
              W           S3
              |           |
          ──N──S1──E──S2──C──
                      |        ‖
                      Y        X
```
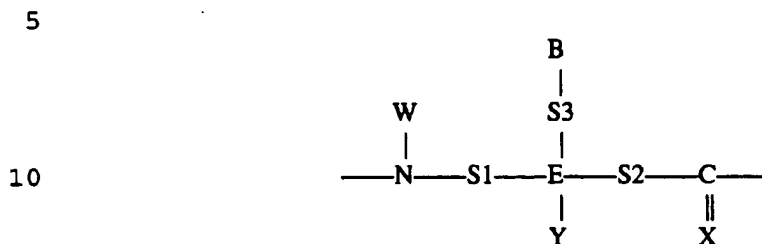
wherein:

E is carbon (C);

W is hydrogen or a spacer group;

Y is hydrogen or a spacer group;

S1 is a bond or a first spacer group;

S2 is a bond or a second spacer group;

S3 is a third spacer group with a backbone of at least two atoms;

X is oxygen (O) or sulfur (S);

B is a base segment comprising a nucleotidic base or an analog thereof; and

N is nitrogen;

and wherein at least one of S1 and S2 is a spacer group, and wherein S3 is a spacer group with a backbone of at least two atoms;

wherein said free NuAA monomer is of the same formula except that the N-terminus is in the form of an amine group or a protected amine group and the C-terminus is in the form of a carboxyl group or a protected carboxyl group.

19.    A method of preparing a PENAM composition of claim 2 for targeting a target nucleic acid comprising:

(a)    providing at least about 4 NuAA monomers wherein at least one of said NuAA monomers comprises a D-chiral center or a quasi-chiral center; and

109

(b)   synthesizing an oligomer of the monomers such
that the arrangement of bases in the oligomer is
substantially complementary to a sequence of nucleotidic
bases in a portion of the target nucleic acid.

5

20.   A method of claim 19, wherein said NuAA
monomers are linked to each other directly via peptide
bonds.

10        21.   A method of claim 19, wherein said NuAA
monomers are linked to each other indirectly via
intervening aminoacyl monomers.

22.   A method of claim 19, further comprising the
15  step of synthesizing a NuAA monomer having a D-chiral
center wherein said NuAA monomer having a D-chiral center
is present at a purity of at least about 90% enantiomeric
excess relative to its enantiomeric NuAA monomer having
an L-chiral center.

20

23.   A method of claim 19, further comprising the
step of synthesizing a NuAA monomer having a quasi-chiral
center.

25        24.   A method of targeting a target nucleic acid
comprising:
(a) providing a PENAM composition according to claim
2; and
(b)   contacting said PENAM composition with the
30  target nucleic acid.

25.  A method of modulating a target nucleic acid in an antisense manner comprising:

(a) providing a PENAM composition according to claim 2 wherein the sequence of bases in said NuAA monomers is 5 substantially complementary to a sequence of bases in the target nucleic acid; and

(b)  contacting  said  PENAM  composition  with  the target nucleic acid.

10       26.   A method of modifying a target nucleic acid comprising:

(a) providing a PENAM composition of claim 2 wherein said homomorphically-preferred PENAM further comprises a target modifying group; and

15       (b)  contacting  said  PENAM  composition  with  the target nucleic acid.

27.   A method of detecting a target nucleic acid comprising:

20       (a) providing a PENAM composition according to claim 2 wherein the sequence of bases in said NuAA monomers is substantially complementary to a sequence of bases in the target nucleic acid; and

(b)  contacting  said  PENAM  composition  with  the 25 target nucleic acid; and

(c)  detecting  a  target  complex  comprising  said nucleic  acid  mimic  and  said  target  nucleic  acid  or detecting a modification in the target nucleic acid.

111

28.   A method of isolating a target nucleic acid comprising:

(a) providing a PENAM composition according to claim 2 wherein the sequence of bases in said NuAA monomers is
5  substantially complementary to a sequence of bases in the target nucleic acid; and

(b) contacting said PENAM composition with the target nucleic acid; and

(c) isolating PENAMs bound to said target nucleic
10  acid or isolating a modified target nucleic acid.
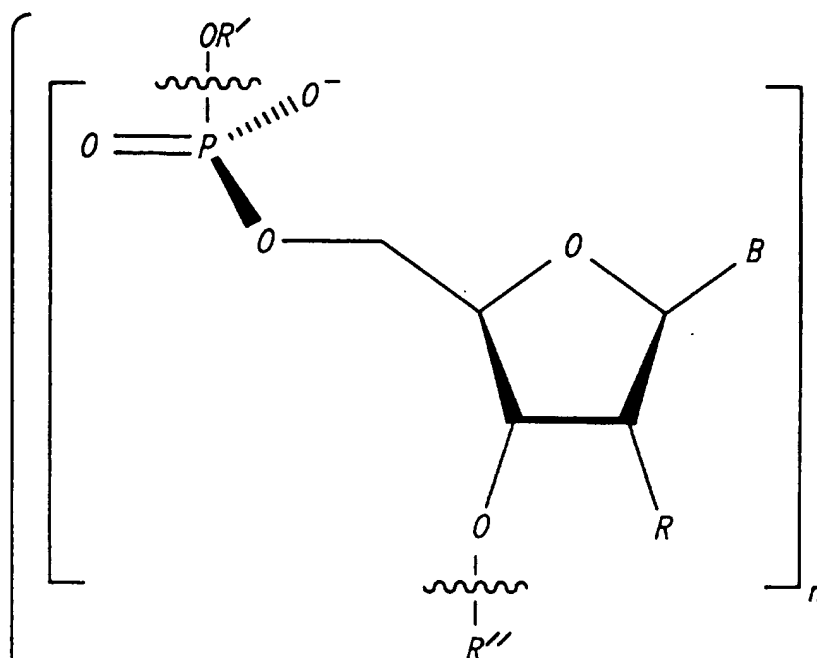
DNA/RNA/ONT
B = A, T, G, C, U
R = H, OH; R′ = R″ = PROTECTIVE GROUPS

*FIG. 1*
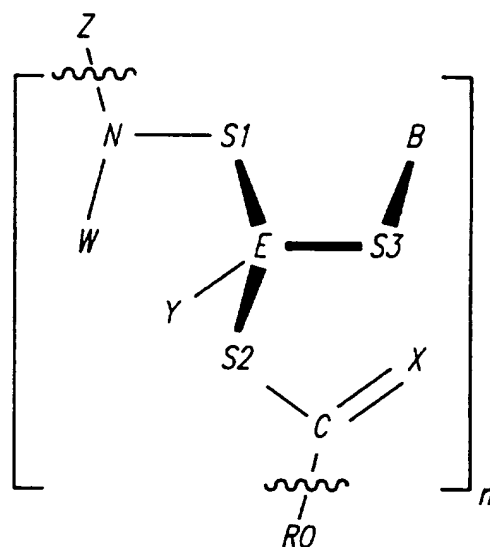
PEPTIDE NUCLEIC ACID MIMIC (PENAM)
E = C, N; X = O, S
B = A, T, G, C, U, & Pu/Py ANALOGS
S1, S2, S3, W, Y = SPACER GROUPS
Z, R = PROTECTIVE GROUPS

*FIG. 2A*

*FIG. 2B*

FIG. 3A

# FIG. 3B

# FIG. 3C

# FIG. 3D

FIG. 3E

*FIG. 4A*

(D/L)-PEPTIDE NUCLEIC ACID MIMIC (PENAM)
Nu = A, T, G, C, U, Pu/Py ANALOGS
Z, R = PROTECTIVE GROUPS; p = e.g. 2 - 3...; n = e.g. 2 - 8...

(D/L)-NUCLEIC AMINOACID (NuAA)
Nu = A, T, G, C, U, Pu/Py ANALOGS
p = e.g. 2 - 3...

(D/L)-HALO AMINOACID (XAA)
X = Br, CI, I, p = e.g. 2 - 3...

(D/L)-ω-HYDROXY AMINOACID
P = e.g. 1 - 3...

(D/L)-HOMOGLUTAMIC ACID

(D/L)-α-AMINO-ω-LACTONE
p = e.g. 1, 2, 3...

(D/L)-ASPARTIC ACID

(D/L)-GLUTAMIC ACID
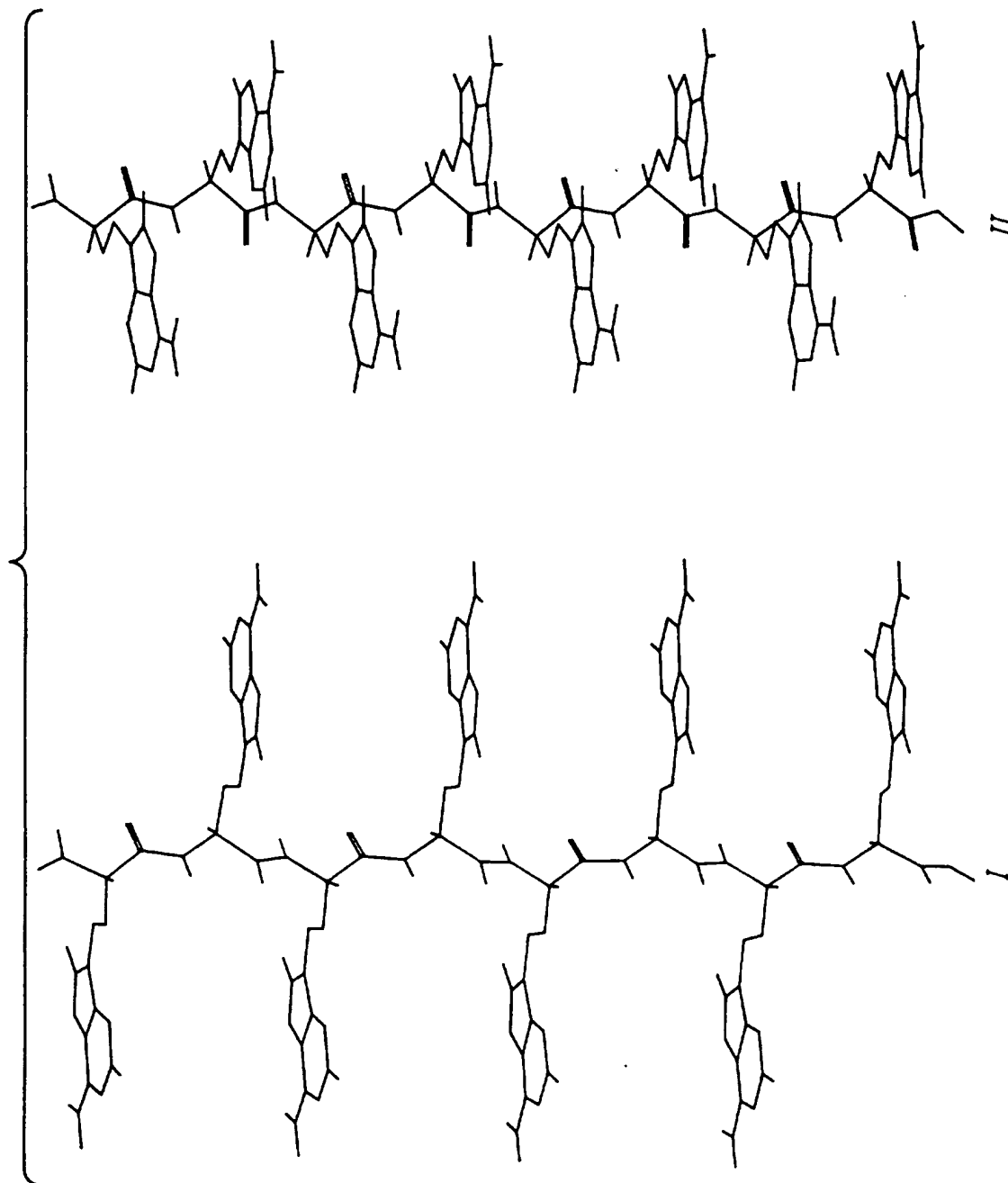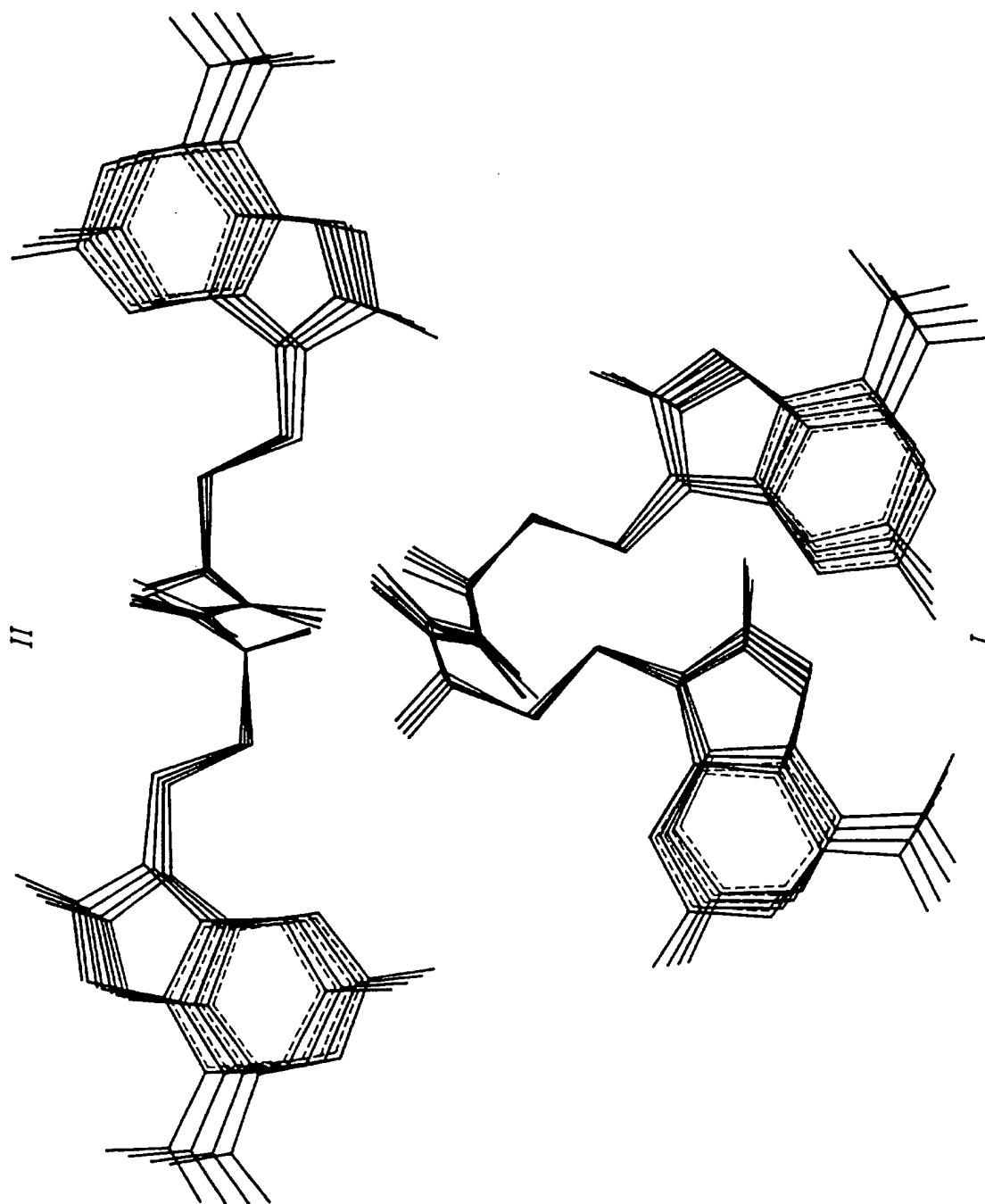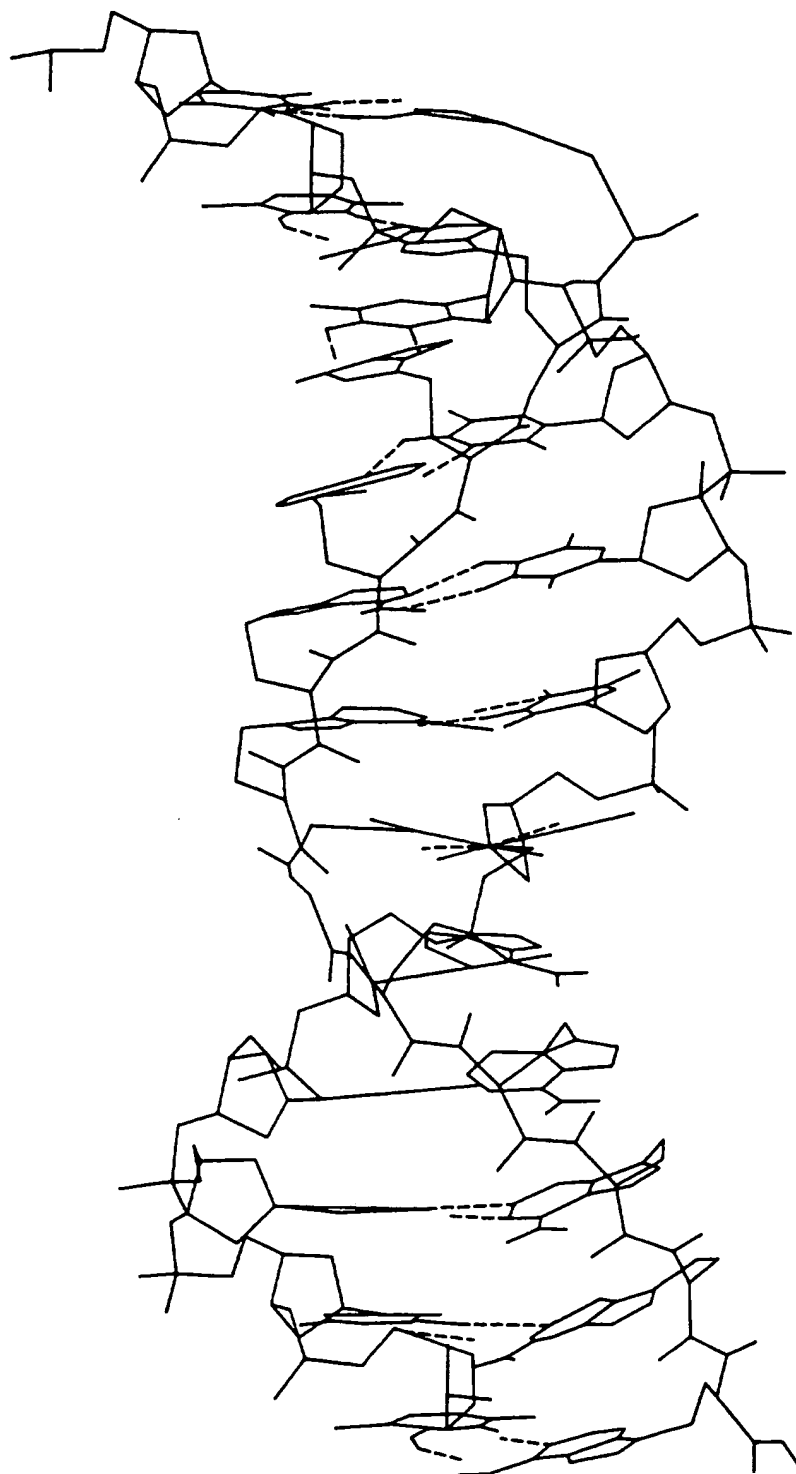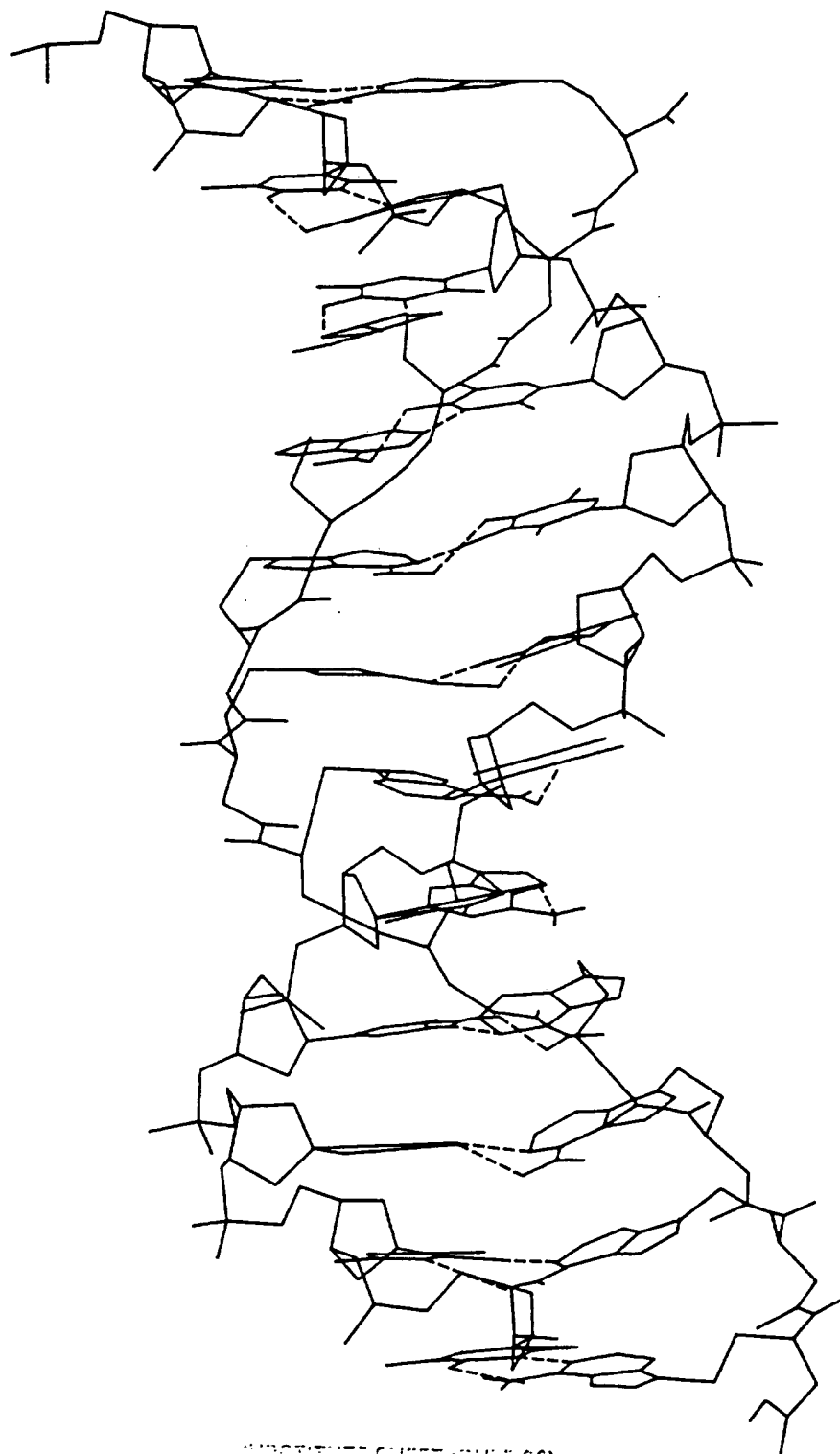
SUBSTITUTE SHEET (RULE 26)

(D/L)-METHIONINE
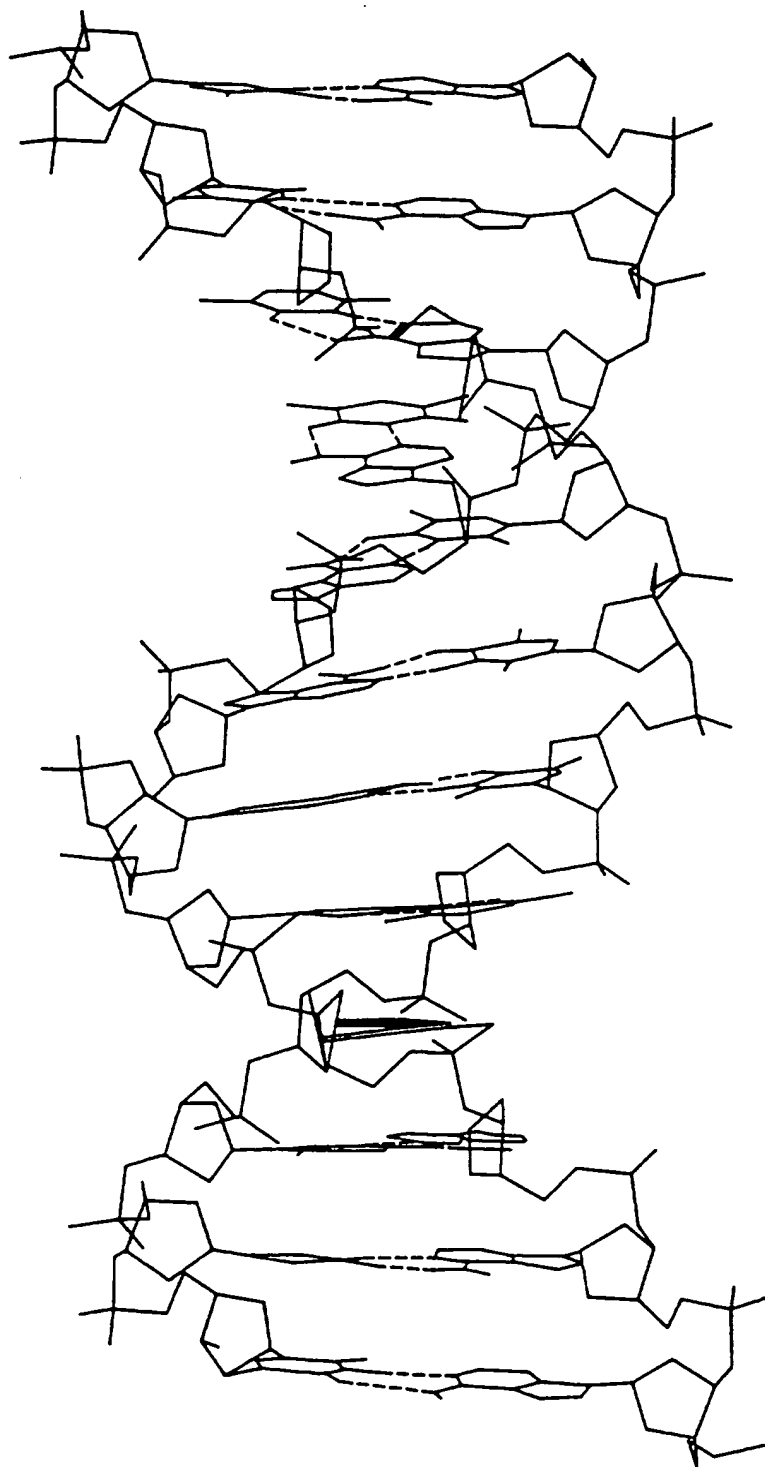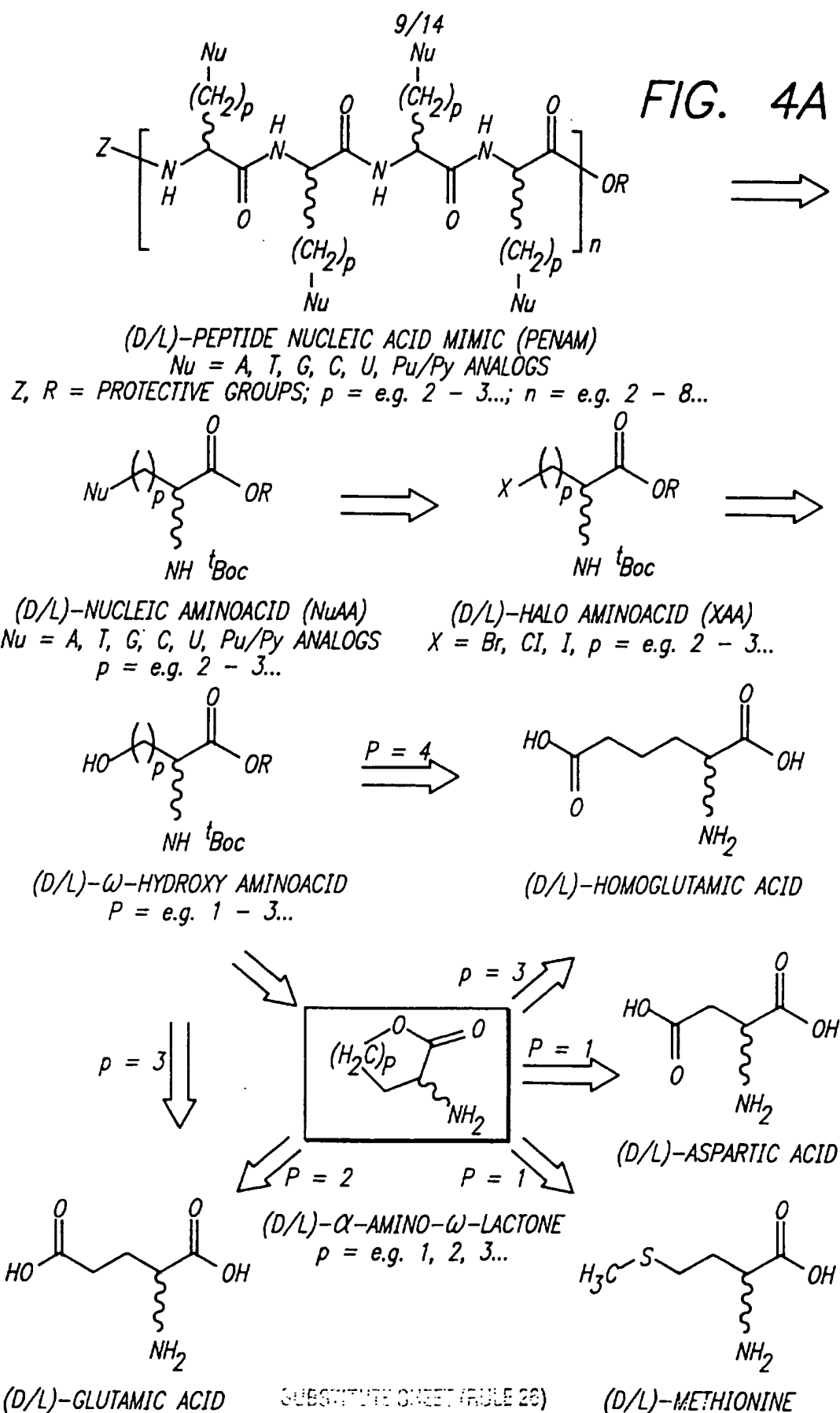
FIG. 4B
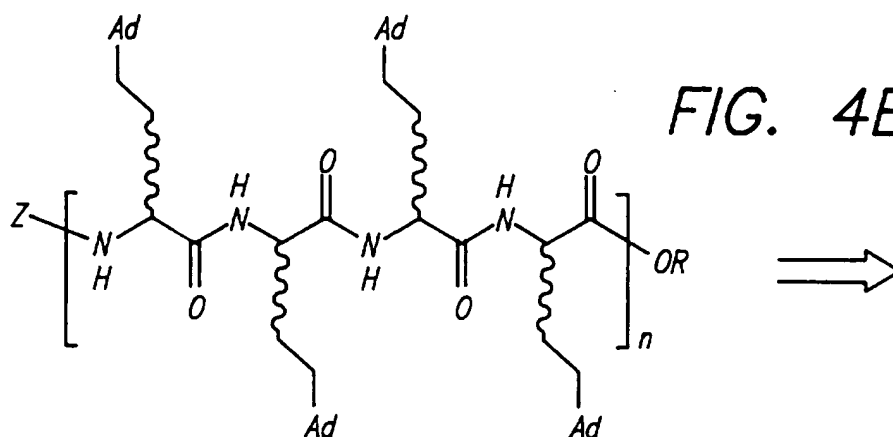
(D/L)-PEPTIDE NUCLEIC ACID MIMIC (PENAM)
Ad = ADENINE
Z, R = PROTECTIVE GROUPS; n = e.g. 2 - 8...
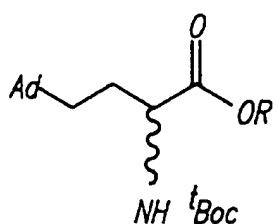
(D/L)-NUCLEICAMINO ACID (AdhAla)
Ad = ADENINE
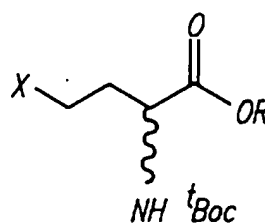
(D/L)-HALOAMINO ACID (XAA)
X = Br, I, CI
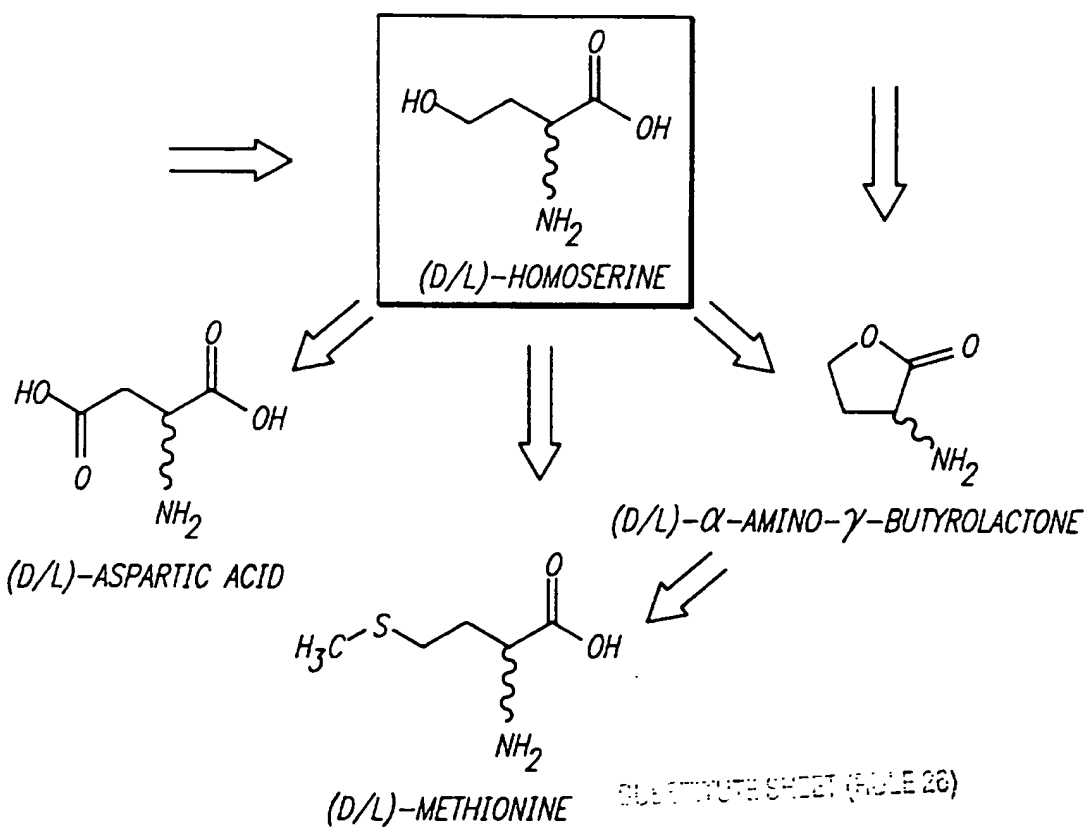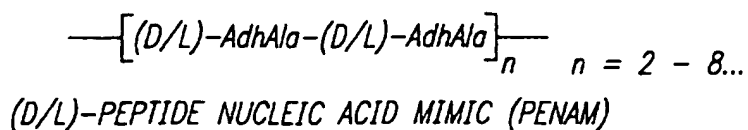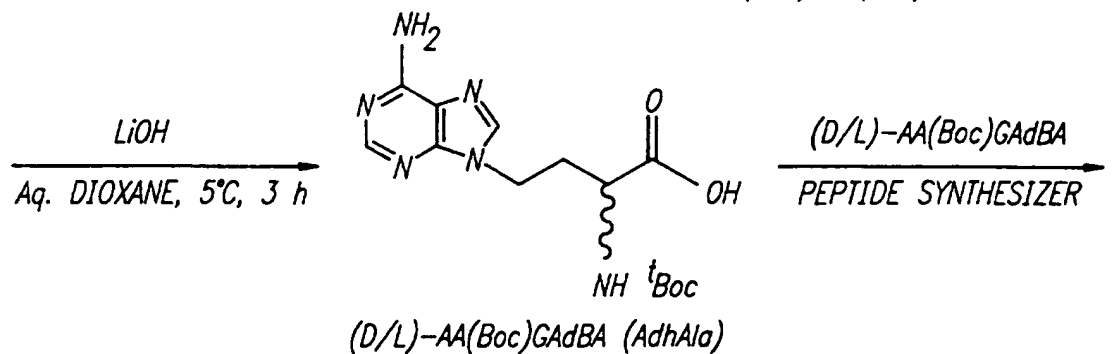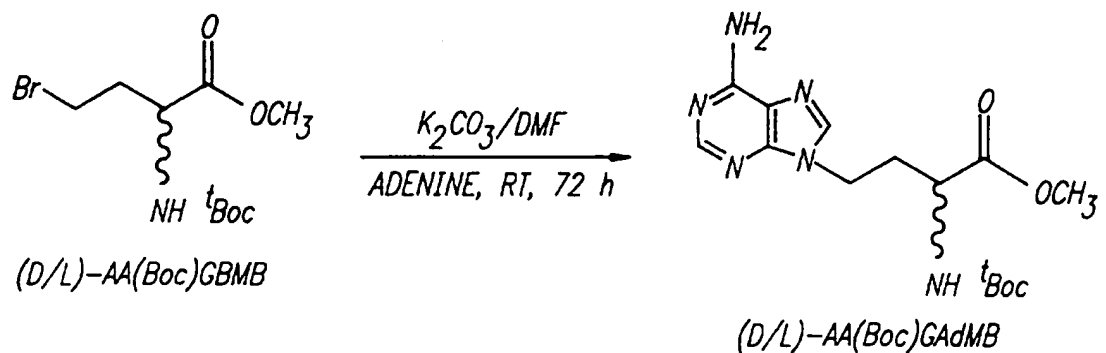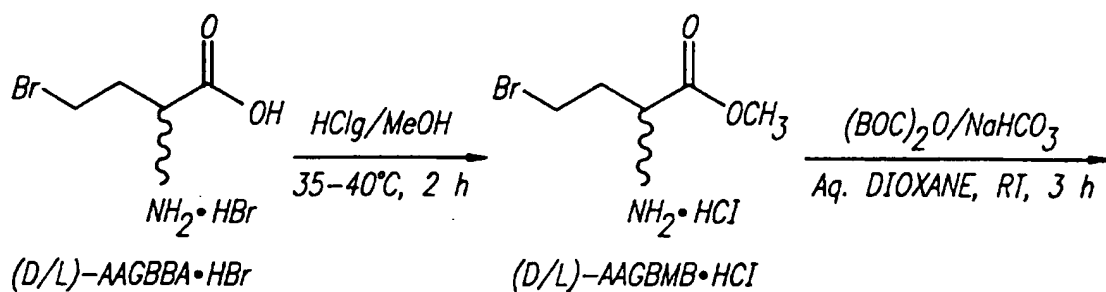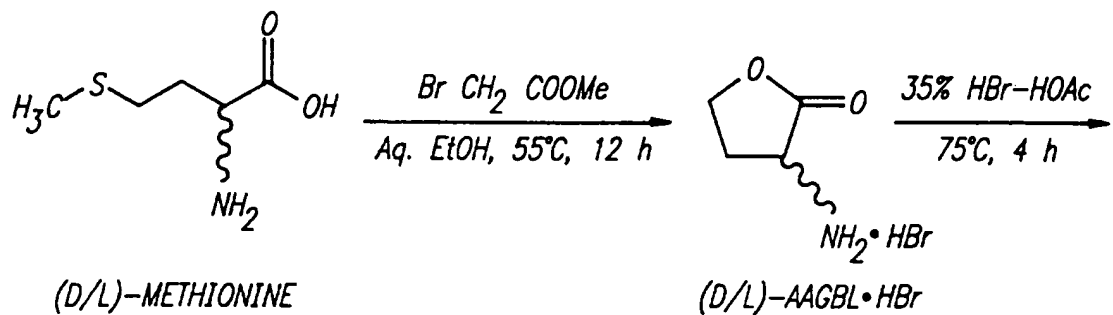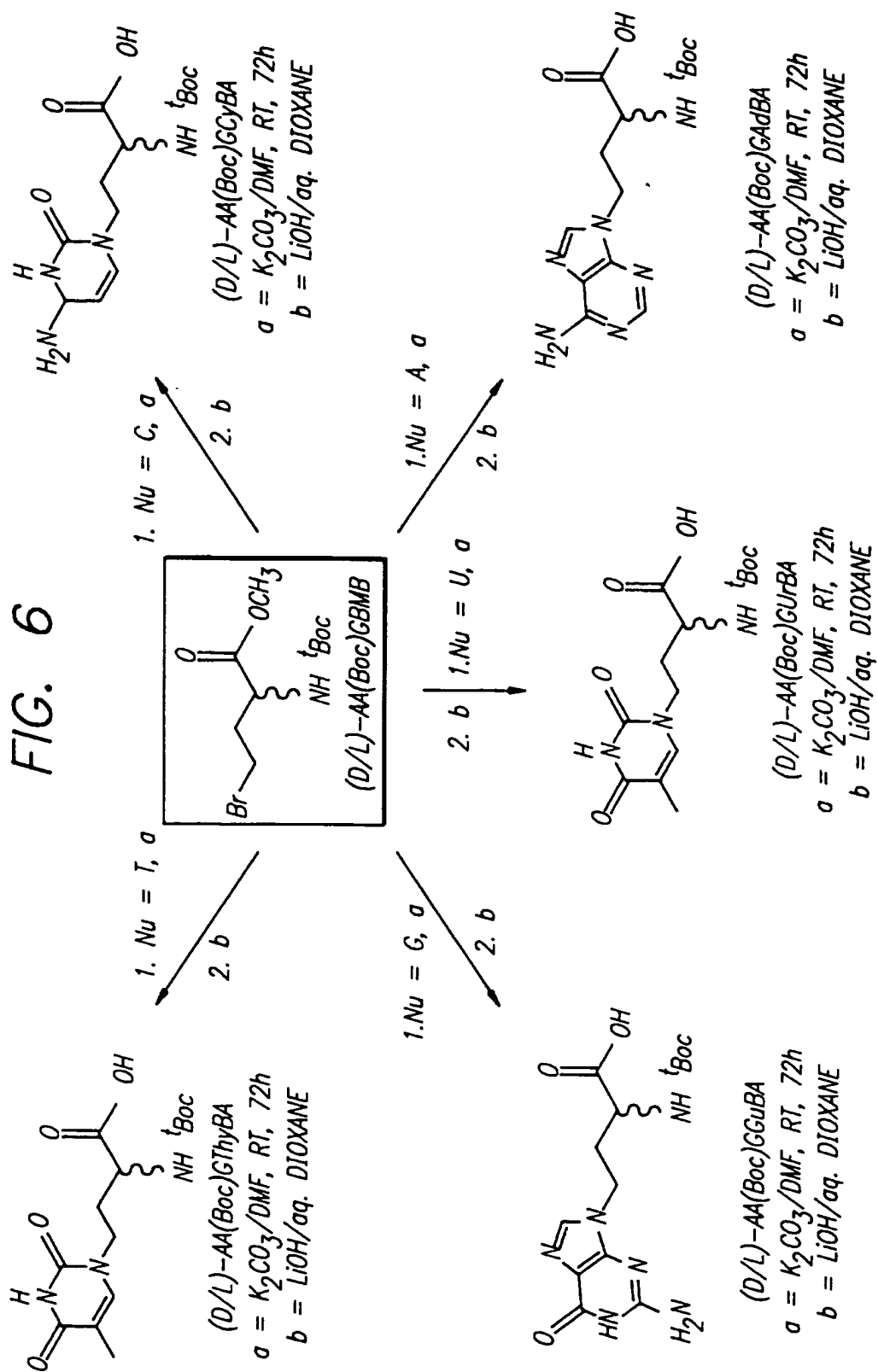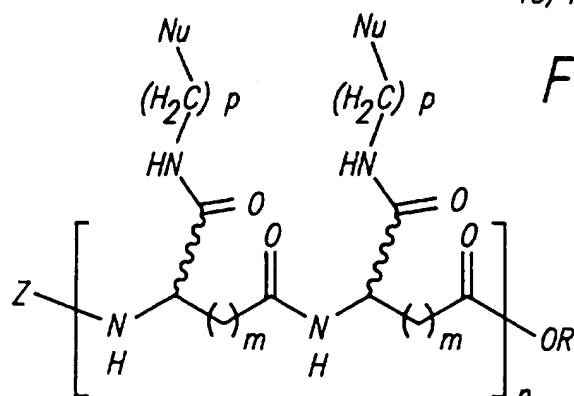
(D/L)-HOMOSERINE

(D/L)-ASPARTIC ACID

(D/L)-α-AMINO-γ-BUTYROLACTONE

(D/L)-METHIONINE

# FIG. 5



(D/L)-METHIONINE

$Br\ CH_2\ COOMe$ / Aq. EtOH, 55°C, 12 h

(D/L)-AAGBL·HBr

35% HBr-HOAc / 75°C, 4 h

(D/L)-AAGBBA·HBr

HClg/MeOH / 35-40°C, 2 h

(D/L)-AAGBMB·HCl

$(BOC)_2O/NaHCO_3$ / Aq. DIOXANE, RT, 3 h

(D/L)-AA(Boc)GBMB

$K_2CO_3/DMF$ / ADENINE, RT, 72 h

(D/L)-AA(Boc)GAdMB

LiOH / Aq. DIOXANE, 5°C, 3 h

(D/L)-AA(Boc)GAdBA (AdhAla)

(D/L)-AA(Boc)GAdBA / PEPTIDE SYNTHESIZER

$\overline{\phantom{xx}}\left[(D/L)\text{-}AdhAla\text{-}(D/L)\text{-}AdhAla\right]_n\phantom{xx}$  n = 2 − 8...

(D/L)-PEPTIDE NUCLEIC ACID MIMIC (PENAM)

FIG. 6

FIG. 7

(D/L)-ω-PEPTIDE NUCLEIC ACID MIMIC (PENAM)
Nu = A, T, G, C, U, Pu/Py ANALOGS
Z, R = PROTECTIVE GROUPS
p = e.g. 1-3... ;
m = e.g. 1-3... ; n = e.g. 2-8...
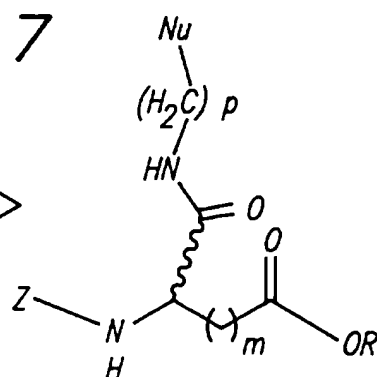
(D/L)-ω-NuAA
Nu = A, T, G, C, U, Pu/Py ANALOGS
Z, R = PROTECTIVE GROUPS
p = e.g. 1-3... ; m = e.g. 1-3...
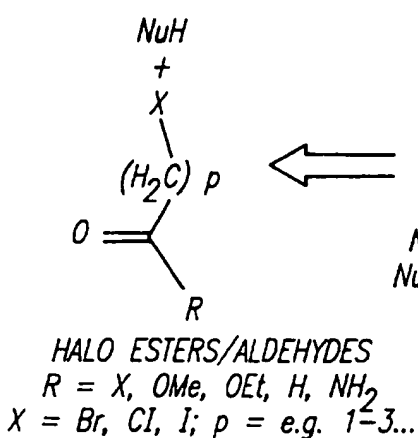
HALO ESTERS/ALDEHYDES
R = X, OMe, OEt, H, $NH_2$
X = Br, CI, I; p = e.g. 1-3...
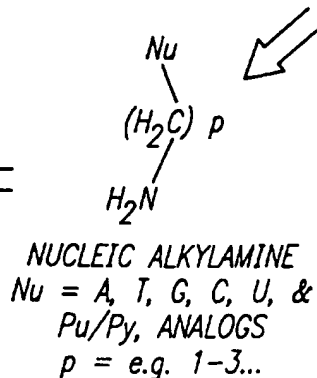
NUCLEIC ALKYLAMINE
Nu = A, T, G, C, U, &
Pu/Py, ANALOGS
p = e.g. 1-3...

(D/L)-GLUTAMIC ACID

m = 2

(D/L)-α-AMINO-ω,α-DICARBOXYBLIC ACID
m = e.g. 1-3...

m = 3

(D/L)-HOMOGLUTAMIC ACID

m = 1
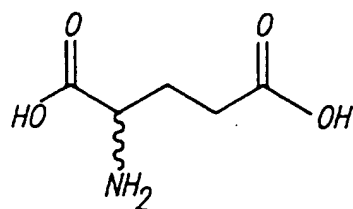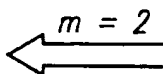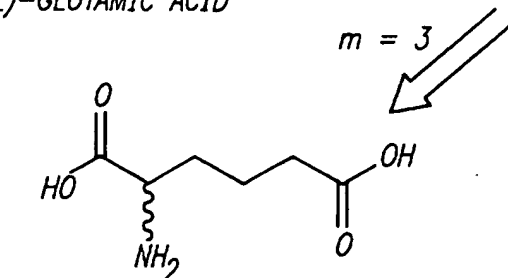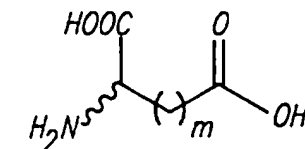
(D/L)-ASPARTIC ACID

14/14
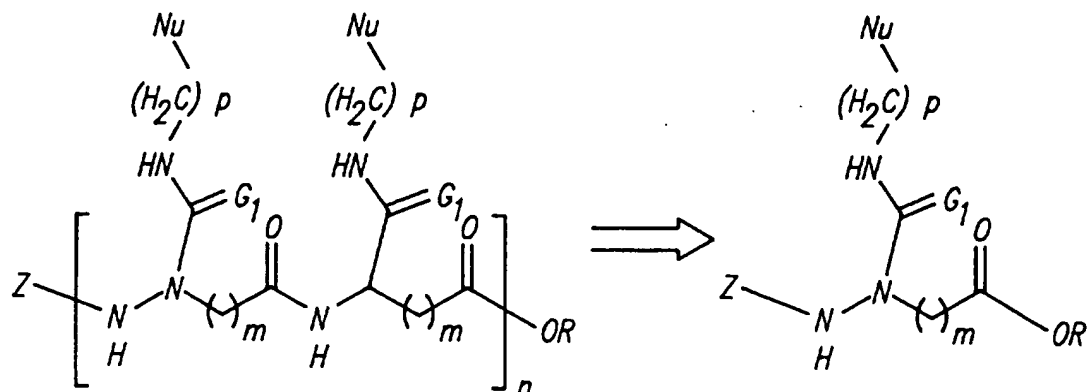


ω−azaPEPTIDE NUCLEIC ACID MIMIC (azaPENAM)
Nu = A, T, G, C, U, Pu/Py ANALOGS
Z, R = PROTECTIVE GROUPS;
G1 = O, S, H2; p = e.g. 1−3... ;
m = e.g. 0−3... ; n = e.g. 2−8...

ω−NuAzaAA
Nu = A, T, G, C, U, Pu/Py ANALOGS
Z, R = PROTECTIVE GROUPS;
G1 = O, S, H2
p = e.g. 1−3... ; m = e.g. 0−3...
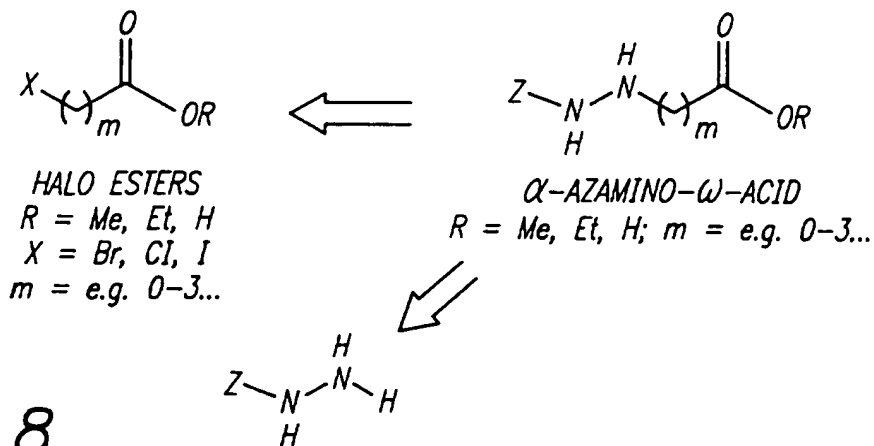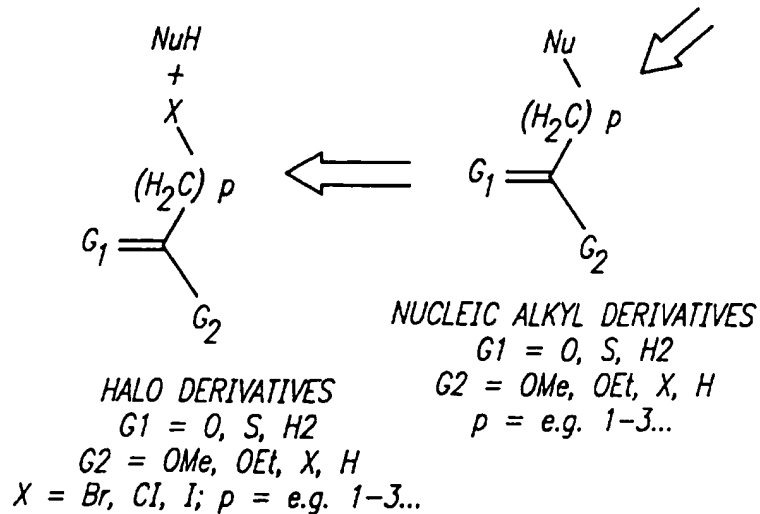
HALO DERIVATIVES
G1 = O, S, H2
G2 = OMe, OEt, X, H
X = Br, Cl, I; p = e.g. 1−3...

NUCLEIC ALKYL DERIVATIVES
G1 = O, S, H2
G2 = OMe, OEt, X, H
p = e.g. 1−3...

HALO ESTERS
R = Me, Et, H
X = Br, Cl, I
m = e.g. 0−3...

α−AZAMINO−ω−ACID
R = Me, Et, H; m = e.g. 0−3...

*FIG. 8*

HYDRAZINE
Z = PROTECTIVE GROUP

# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :536/23.1, 24.5, 25.4; 530/333; 435/6
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.5, 25.4; 530/333; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, MEDLINE
search terms: oligo?, nucleic acid, nucleotide, purine, pyrimidine, peptide, amino(w)acid, mimic

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Journal of the American Chemical Society, Volume 114, issued 1992, R. Zuckermann et al., "Efficient Method for the Preparation of Peptoids [Oligo(N-Substituted Glycines) by Submonomer Solid-Phase Synthesis", pages 10646-10647, see entire document. | 1-25 |
| Y | Nucleic Acids Research, Volume 21, No. 8, issued 1993, D. Ecker et al., "Rational Screening of Oligonucleotide Combinatorial Libraries for Drug Discovery", pages 1853-1856, see entire document. | 1-25 |
| Y | Gene, Volume 149, issued 1994, P. Nielsen et al., "Sequence-Specific Transcription Arrest by Peptide Nucleic Acid Bound to the DNA Template Strand", pages 139-145, see entire document. | 1-25 |

[X] Further documents are listed in the continuation of Box C.    [ ] See patent family annex.

| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
|---|---|---|---|
| "A" | document defining the general state of the art which is not considered to be of particular relevance | | |
| "E" | earlier document published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 14 NOVEMBER 1995 | 20 DEC 1995 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br>CHARLES C. P. RORIES |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

# INTERNATIONAL SEARCH REPORT

**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Advances in Polymer Science, Volume 41, issued 1981, K. Takemoto et al., "Synthetic Nucleic Acid Analogs, Preparation and Interaction", pages 1-51, see entire document. | 1-25 |
| Y | Journal of the American Chemical Society, Volume 114, issued 1992, M. Egholm et al., "Peptide Nucleic Acids (PNA). Oligonucleotide Analogues with an Achiral Peptide Backbone", pages 1895-1897, see entire document. | 1-25 |
| Y | Science, Volume 354, issued 06 December 1991, P. Nielsen et al., "Sequence-Selective Recognition of DNA by Strand Displacement with a Thymine-Substituted Polyamide", pages 1497-1500, see entire document. | 1-25 |
| Y | Science, Volume 258, issued 27 November 1992, J. Hanvey et al., "Antisense and Antigene Properties of Peptide Nucleic Acids", pages 1481-1485, see entire document. | 1-25 |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/09828

| Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |
| --- |

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |
| --- |

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable
   claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers
   only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-25

**Remark on Protest**
   ☐ The additional search fees were accompanied by the applicant's protest.
   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

## A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/00; C07D 237/00, 473/00; C07H 21/00; C07K 1/00, 2/00; C12Q 1/68

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-25, drawn to a composition comprising a peptide-based nucleic acid mimic (PENAM), a monomeric precursor of a PENAM, a method for making a PENAM, and a method for using a PENAM as an antisense inhibitor of nucleic acid metabolism, classified in Class 536, subclass 24.5, for example.

II. Claim 26, drawn to a method for modifying the chemical structure of a target nucleic acid, classified in Class 435, subclass 91.1, for example.

III. Claim 27, drawn to a method for detecting a nucleic acid, classified in Class 435, subclass 6, for example.

IV. Claim 28, drawn to a method for isolating a nucleic acid, classified in Class 536, subclass 25.4, for example.

The claims lack unity of invention under 37 C.F.R. 1.475(d), as the claims of Groups II, III, and IV are each drawn to a separate method for using the disclosed antisense nucleic acid mimic that is materially different from the first-to-be claimed method for using the antisense nucleic acid mimic to inhibit nucleic acid metabolism which is included in Group I.